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Plant Signal Molecules Activate the *syrB* Gene, Which Is Required for Syringomycin Production by *Pseudomonas syringae* pv. syringae†

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The *syrB* gene is required for syringomycin production by *Pseudomonas syringae* pv. syringae and full virulence during plant pathogenesis. Strain B3AR132 containing a *syrB*::*lacZ* fusion was used to detect transcriptional activation of the *syrB* gene in syringomycin minimal medium by plant metabolites with signal activity. Among 34 plant phenolic compounds tested, arbutin, phenyl-β-D-glucopyranoside, and salicin were shown to be strong inducers of *syrB*, giving rise to ~1,200 U of β-galactosidase activity at 100 μM; esculin and helicin were moderate inducers, with about 250 to 400 U of β-galactosidase activity at 100 μM. Acetosyringone and flavonoids that serve as signal molecules in *Agrobacterium* and *Rhizobium* species, respectively, did not induce the *syrB*::*lacZ* fusion. All *syrB* inducers were phenolic glucosides and none of the aglucone derivatives were active, suggesting that the β-glycosidic linkage was necessary for signal activity. Phenyl-β-D-galactopyranoside containing galactose substituted for glucose in the β-glycosidic linkage also lacked inducer activity. Phenolic signal activity was enhanced two- to fivefold by specific sugars common to plant tissues, including D-fructose, D-mannose, and sucrose. The effect of sugars on *syrB* induction was most noticeable at low concentrations of phenolic glucoside (i.e., 1 to 10 μM), indicating that sugars such as D-fructose increase the sensitivity of *P. syringae* pv. syringae to the phenolic plant signal. Besides induction of *syrB*, syringomycin biosynthesis by parental strain B3A-R was induced to yield over 250 U of toxin by the additions of arbutin and D-fructose to syringomycin minimal medium. These data indicate that syringomycin production by most strains of *P. syringae* pv. syringae is modulated by the perception of two classes of plant signal molecules and transduced to the transcriptional apparatus of syringomycin (*syr*) genes such as *syrB*.

Most phytopathogenic strains of *Pseudomonas syringae* pv. syringae produce a cyclic lipodepsinonapeptide toxin called syringomycin (12, 30). It is a potent phytotoxin that causes necrotic symptoms by disrupting ion transport across the plasmalemma of affected cells (2, 27). Accordingly, syringomycin production is an important element of virulence in *P. syringae* pv. syringae, a pathogen of numerous dicot and monocot species (3). A nontoxigenic (Tox^-) mutant of strain B301D-R resulting from insertion of Tn*5* into the *syrB* gene caused only small lesions in immature sweet cherry (*Prunus avium* L.) fruits (36, 37). Toxigenicity was estimated to nearly double the virulence of *P. syringae* pv. syringae based on quantitative evaluations of disease caused by the *syrB* mutant compared with the parental strain or a restored strain obtained by marker exchange of the intact *syrB* gene.

A vital role in syringomycin synthesis is attributed to the *syrB* gene. We recently observed that two of the large proteins associated with syringomycin production, SR4 and SR5 (~350 and ~130 kDa, respectively), were deficient in *syrB* mutants obtained by Tn*3*HoHo1 mutagenesis (24). The proteins are postulated to function as part of the syringomycin synthetase complex that likely resembles the thioltemplate multienzyme mechanism responsible for the formation of the structurally related polypeptin and polymyxin antibiotics produced by *Bacillus* spp. (18, 32). The *syrB* locus was estimated to be 3.1 kb based on mapping analysis of Tn*3*HoHo1 insertions (24). Consequently, it was surmised that *syrB* encodes either a subunit of a multimeric synthetase protein (i.e., SR4, SR5, or both) or a positive regulatory protein which controls synthetase expression.

The environmental conditions required for expression of the *syrB* gene correspond with those permissive to syringomycin production. Tn*3*HoHo1 mutagenesis (34) of *syrB* was used to obtain random transcriptional fusions to a promoterless *lac* operon, and a *syrB*::*lacZ* fusion that expressed high β-galactosidase activity was recombined into the chromosome of strains B301D-R and B3A-R (rifampin-resistant derivatives of different wild isolates, i.e., B301D and B3A) to generate BR132 and B3AR132, respectively (24). Iron concentration had a positive regulatory effect on the expression of the *syrB*::*lacZ* fusion in both strains similar to the iron regulatory effects on toxin production (11). The temporal expression of *syrB* also conformed to that of syringomycin biosynthesis, with peak activity occurring after 3 to 4 days of incubation (24). In accordance with antibiotic biosynthesis in many microorganisms, syringomycin production is initiated when cells enter the stationary phase and corresponds to the formation of large proteins hypothesized to function as synthetases (24, 25). However, the two strains showed differential expression in culture media depending on the presence or absence of a plant extract. In a medium containing an extract from potato (i.e., potato dextrose broth [PDB]), both BR132 and B3AR132 expressed high β-galactosidase activity, whereas in a defined medium (i.e., syringomycin minimal [SRM]), only strain BR132 was active. The failure of the *syrB*::*lacZ* fusion in B3AR132 to be expressed

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in SRM medium also was consistent with a lack of toxin production in SRM medium by parental strain B3A-R. Two lines of evidence suggested that the *syrB*::*lacZ* fusion in B3AR132 was plant inducible (24). First, significant β-galactosidase activity was recovered from immature cherry fruits inoculated with B3AR132; over 400 U were recovered only 24 h after inoculation. Second, addition of a crude aqueous extract from cherry leaves to SRM medium induced appreciable β-galactosidase activity in B3AR132, suggesting that a nonnutritive constituent(s) functioned as a signal molecule perceived by the bacterium and transduced to the *syrB* transcriptional apparatus.

An emerging concept in plant-bacterium associations is the central role of plant phenolic compounds as regulators of gene expression in phytobacteria (29). For example, several virulence (*vir*) genes in *Agrobacterium tumefaciens* are transcriptionally activated after perception of particular phenolic signal molecules such as acetosyringone in the wound environment (20, 33, 35). In *Rhizobium* spp., specific plant flavonoids are required for induction of nodulation (*nod*) genes (13). Host-specific nodulation is primarily mediated via the *nodD* symbiotic regulatory gene that transduces an appropriate flavonoid signal by controlling the expression of other *nod* genes. An analogous system of plant phenolic signals responsible for transcriptional activation of genes critical to the plant-microbe interaction remains to be demonstrated for other groups of phytobacteria such as *P. syringae*.

A second class of *vir* gene-inducing compounds was recently identified in *A. tumefaciens* to be composed of simple pyranose sugars such as D-fucose, D-galactose, D-glucose, and D-mannose (1, 5, 31). These saccharides act by markedly enhancing *vir* gene induction when low concentrations of the phenolic signal molecule are present. For example, a *virB*::*lacZ* fusion was induced 60- to 200-fold more when a 10 mM concentration of an inducing sugar was added to a glycerol-based medium containing 2.5 μM of acetosyringone (5). The sugar-mediated induction requires the ChvE protein, which functions as a periplasmic galactose-glucose-binding protein in *A. tumefaciens* that is believed to interact with the periplasmic domain of the VirA protein to signal *vir* gene induction.

In this study, we explored the possibility that plant phenolic compounds and saccharides synergistically interact to control expression of the *syrB* gene and syringomycin production in some strains of *P. syringae* pv. syringae such as B3A-R. A number of commercially available plant phenolic compounds and sugars were tested with a *syrB*::*lacZ* fusion that is known to be expressed in B3AR132 only in an environment containing undefined plant metabolites. Two classes of plant metabolites were shown to function as signal molecules in *P. syringae* pv. syringae. Evidence is presented that certain phenolic glucosides serve as the primary signal and that activity is enhanced by the presence of specific mono- and disaccharides. We also demonstrate that a phenolic signal molecule, arbutin, together with a sugar signal molecule, D-fructose, induces syringomycin production in parental strain B3A-R. The chemical requirements for signal activity are discussed for *P. syringae* pv. syringae and compared with signal transduction processes in the members of the family *Rhizobiaceae*.

MATERIALS AND METHODS

Bacterial strains and media. The wild-type strains B301D, B3A, and HS191 of *P. syringae* pv. syringae produced large quantities of syringomycin on potato dextrose agar and were described previously (6, 10, 11). Strains B301D-R and B3A-R are spontaneous rifampin-resistant (100 μg/ml) mutants of B301D and B3A, respectively (24). Strain B301D-R produces syringomycin in both SRM and PDB media, whereas strains B3A-R and HS191 produce syringomycin only in PDB medium (11). The *syrB* gene was cloned from strain B301D-R (24). The Tox$^-$ strain B3AR132 was obtained by recombination of an in-frame *syrB*::*lacZ* fusion, generated by Tn*3*HoHo1 mutagenesis (34), into the chromosome of parental strain B3A-R by marker exchange as described by Mo and Gross (24). A second Tox$^-$ strain, B3AR253, carried a *syrB*::*lacZ* fusion oriented opposite to the direction of transcription of *syrB*. Of the two *syrB* mutant strains, only B3AR132 expressed β-galactosidase activity under conditions conducive to syringomycin production (24).

King's medium B (KB) agar (17) was used for routine culture of all strains except that rifampin (Sigma Chemical Co., St. Louis, Mo.) was added (50 μg/ml) when strains B301D-R and B3A-R were cultured. The *syrB*::*lacZ* mutant strains, B3AR132 and B3AR253, were grown on KB agar supplemented with piperacillin (Sigma) at 25 μg/ml. Potato dextrose agar and PDB and SRM media were as described previously (11). SRM medium contains 1.0% D-glucose, 0.4% L-histidine, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.8 mM potassium phosphate, and 10 μM $FeCl_3$ ($FeCl_3$ was added to SRM medium immediately before use to ensure an adequate supply of available Fe^{3+} for maximum expression of *syrB* or syringomycin production). Complete SRM medium was used as the basal medium in all tests of phenolic compounds, sugars, and organic acids for *syrB*-inducing activity or induction of syringomycin production. Methods for long-term preservation of strains were described earlier (36).

Syringomycin production. The bioassay with the fungus *Geotrichum candidum* F-260 was used to determine the yield of syringomycin in liquid cultures (50 ml) (12). Syringomycin production was tested in strains B301D-R, B3A-R, and HS191 grown in PDB, SRM medium, SRM medium plus fructose (0.1%), SRM medium plus arbutin (100 μM), and SRM medium plus arbutin (100 μM) and fructose (0.1%). Duplicate cultures were prepared for each treatment.

Phenolic compounds, sugars, and organic acids tested for *syrB* induction. Amygdalin, apigenin, arbutin, catechin, chlorogenic acid, epicatechin, esculetin, esculin, fraxin, hydroquinone, hydroxybenzoic acid, naringenin, naringin, phenol, prunasin, quercetin, quinic acid, rutin, salicin, saligenin, and vanillic acid were purchased from Sigma. Flavone, flavonone, helicin, hesperidin, methylchalcone, 3-hydroxyflavone, 7-hydroxyflavone, and rhapontin were purchased from Aldrich Chemical Co., Milwaukee, Wis. Hesperidin, 4-methylumbelliferyl-glucopyranoside, phenyl-β-D-galactopyranoside, phenyl-β-D-glucopyranoside, and phloridzin were purchased from Fluka Chemical Corp., Ronkonkoma, N.Y. Acetosyringone was purchased from Jansen Life Science, Beerse, Belgium.

All sugars (L-arabinose, D-cellobiose, D-fructose, D-fucose, L-galactose, D-glucose, α-lactose, lactulose, maltose, D-mannose, L-mannose, D-raffinose, L-rhamnose, D-ribose, sucrose, and D-xylose), sugar alcohols (*myo*-inositol, D-mannitol, and D-sorbitol), sodium citrate, and pyruvate were purchased from Sigma, Fluka, or the J. T. Baker Chemical Co., Phillipsburg, N.J.

Preparation of inocula for tests of *syrB* induction. Inocula of strains B3AR132 or B3AR253 used in routine tests of *syrB* induction were prepared from cells grown for 3 days in SRM

liquid. Single colonies grown on SRM agar for 48 h were transferred to 25 ml of SRM medium and incubated overnight at 25°C with rotary shaking (250 rpm). After incubation overnight, the cells were further incubated without shaking for 3 days. Bacterial cells were collected by centrifugation and resuspended in SRM medium containing 15% glycerol (vol/vol) to give about 5×10^8 CFU/ml. Cell stocks suspended in 0.5 ml of SRM-glycerol medium were stored at −80°C for up to 1 month before use in *syrB* induction assays. Assays routinely used 10 μl of the cell stock per 5 ml of SRM medium.

Assays for *syrB* gene induction. The method described by Miller (21), as modified by Stachel et al. (34), was used to measure β-galactosidase activity resulting from expression of a *syrB*::*lacZ* fusion in *P. syringae* pv. syringae. Routinely, 10 μl of inoculum for either strain B3AR132 or B3AR253 was added to 5 ml of SRM medium containing test compounds (phenolic compounds or sugars) to give a final cell density of ~10^6 CFU/ml. Cultures were incubated for 3 days at 25°C without shaking. After incubation, 0.5 ml of each culture was centrifuged and washed once in Z-buffer (21) and then resuspended in 1.4 ml of Z-buffer. Cell densities were measured at 600 nm. Cells contained within a 0.5-ml portion of the suspension were lysed by adding 20 μl each of 0.05% sodium dodecyl sulfate and chloroform and vortexed for ~10 s. Lysed cells were preincubated for 10 min at 28°C and then assayed for β-galactosidase activity by adding 100 μl of freshly prepared *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) (Sigma). After a 10-min incubation, 250 μl of 1 M Na_2CO_3 was added to terminate the reaction. The A_{420} of the solution was measured after the cell debris were pelleted by centrifugation. β-Galactosidase activity was calculated by the formula of Stachel et al. (34). Duplicate cultures were prepared for all treatments; all assays were repeated two or more times on separate days.

All phenolic compounds tested for possible *syrB*-inducing activity were prepared as sterile stock solutions of 1, 10, and 100 mM. Those phenolic compounds that were not highly soluble in water were dissolved in methanol or dimethyl sulfoxide. As controls for possible solvent effects on *syrB* induction, 5-μl volumes of water, methanol, or dimethyl sulfoxide lacking added phenolic compounds were added to cultures. Phenolic compounds were tested at final concentrations of 1, 10, and 100 μM (added as 5 μl of stock solution to 5 ml of an SRM cell suspension) except for arbutin, phenyl-β-D-glucopyranoside, salicin, and esculin, which also were tested at 0.1 and 1,000 μM.

The effects of sugars, sugar alcohols, citrate, and pyruvate on the induction of *syrB* were measured at a final concentration of 0.1% (wt/vol) in SRM medium without or supplemented with arbutin (10 μM). Duplicate cultures of strain B3AR132 were prepared for each treatment; inoculum preparation, culture conditions, and β-galactosidase assays were as described above. Treatments were tested on at least two occasions. In addition, the effect of arbutin concentration (tested at 0.1, 1, 10, 100, and 1,000 μM) on *syrB* expression was determined in SRM medium supplemented with 0.1% (wt/vol) fructose. Similarly, D-fructose (0.1%) also was tested in combination with each of the other phenolic compounds at 10 or 100 μM for *syrB* induction.

Hydroquinone and saligenin were tested in SRM medium at concentrations of 10, 100, and 500 μM for inhibitory effects on *syrB* induction in the presence of arbutin (100 μM). Media were inoculated with strain B3AR132 prepared as described above, and the still cultures were incubated at 25°C for 3 days. Duplicate cultures were prepared for each treatment, and the experiment was repeated on three occasions. After incubation, cell densities and β-galactosidase activities were determined as above for duplicate samples from each culture. Differences in β-galactosidase activity were determined for cultures incubated with or without a phenolic aglucone.

β-Glucosidase assays. Strains B3AR132 and B3AR253 grown in the presence of arbutin were assayed for β-glucosidase activity by a modification of the method of Lee (19) that measures only β-glucosidase activity secreted from cells. SRM medium or SRM medium plus 0.1% D-fructose was supplemented with arbutin to a final concentration of 0.1, 1, 10, 100, or 1,000 μM. The 5-ml cultures were inoculated and incubated as described above. Duplicate cultures were prepared for each treatment, and the experiment was repeated three times. After incubation, the cell densities of the cultures were measured at 600 nm for duplicate samples from each culture. β-Glucosidase assays were conducted by adding 20 μl of sodium citrate (1 M, pH 4.6) to 380 μl of bacterial culture. After preincubation at 30°C for 5 min, the reaction was started by adding 100 μl of *p*-nitrophenyl-β-D-glucopyranoside (Sigma) (25 mM in 0.05 M sodium citrate [pH 4.6]) and incubated at 30°C for 15 min. The reaction was terminated by adding 700 μl of Na_2CO_3 (0.2 M). Cell debris were removed by centrifugation in a microcentrifuge, and the A_{400} was measured. β-Glucosidase activities were determined for duplicate samples from each culture by the formula used to calculate β-galactosidase activity (34).

RESULTS

Induction of *syrB*. Several categories of phenolic compounds initially were surveyed at a concentration of 100 μM in SRM liquid medium for their ability to induce expression of the *syrB*::*lacZ* fusion in strain B3AR132. The categories of phenolic compounds tested included phenolic β-glucosides (arbutin, esculin, and salicin), flavones (apigenin, flavone, 3-hydroxyflavone, and 7-hydroxyflavone), flavonones (naringenin and naringin), flavonols (quercetin and rutin), an acetophenone (acetosyringone), and other phenolic compounds commonly found in plant tissues (catechin, chlorogenic acid, epicatechin, hydroxybenzoic acid, quinic acid, and vanillic acid). The three phenolic β-glucosides induced high levels of β-galactosidase activity ranging from 35- to 100-fold above the background level (~11 U) in SRM medium. None of the other phenolic compounds tested were active based on the recovery of <20 β-galactosidase units.

Once it was established that certain phenolic β-glucosides highly induced the expression of *syrB*, the length of incubation appropriate for maximum activity was determined. Arbutin was used in these experiments because it was the most active of the three β-glucosides. The incubation time required for maximum induction of the *syrB*::*lacZ* fusion in strain B3AR132 by arbutin (100 μM) was determined by using cells previously grown for 3 days in SRM liquid medium (Fig. 1). β-Galactosidase activity increased to over 200 U within 24 h of incubation after the addition of arbutin. By day 3, a maximum activity of over 1,400 U was observed that was maintained to the end of the 5-day incubation period. In contrast, no activity was observed for strain B3AR253, which contained a *lacZ* fusion to *syrB* oriented opposite to the direction of transcription (data not shown). Similar levels of *syrB* expression were observed if cells grown in SRM medium for 16 h were used as the inoculum (late log phase), but peak activities were delayed by 24 to 48 h. Peak β-galactosidase activities were reached with cultures

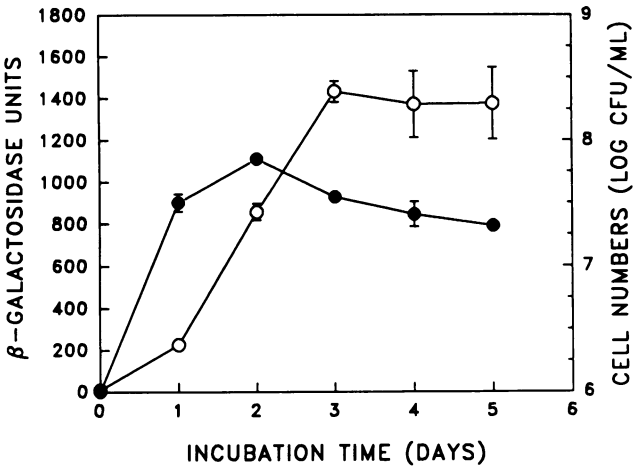

FIG. 1. Relationship between length of incubation and expression of β-galactosidase activity for *syrB*::*lacZ* strain B3AR132 (○) of *P. syringae* pv. syringae grown in SRM liquid medium supplemented with 100 μM of arbutin. Cells initially were cultured in SRM liquid medium and then adjusted to a final concentration of 10^6 CFU/ml in the arbutin-containing SRM medium before incubation as still cultures at 25°C. Changes in total cell numbers (●) are shown for strain B3AR132 over a 5-day incubation. Each value is the mean of three trials conducted on separate occasions; each trial consisted of duplicate cultures. Vertical bars indicate the standard error of the mean.

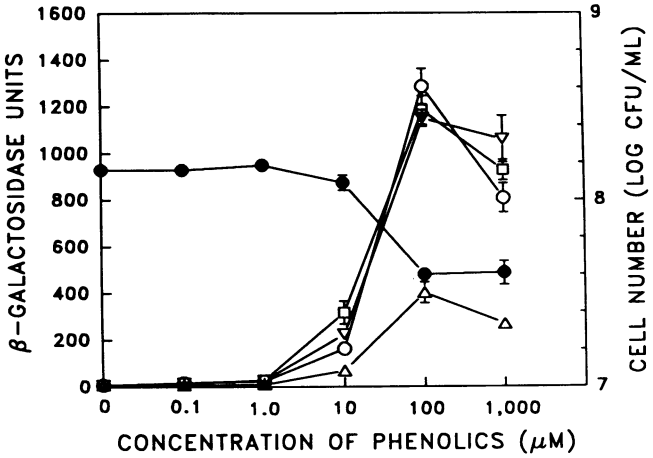

FIG. 2. Effect of concentration of phenolic β-glucosides on induction of the *syrB*::*lacZ* fusion in strain B3AR132 of *P. syringae* pv. syringae. β-Galactosidase activities were measured for strain B3AR132 after incubation for 3 days in SRM liquid medium supplemented with 0.1 to 1,000 μM of arbutin (○), phenyl-β-D-glucopyranoside (□), salicin (▽), or esculin (△). Cell numbers for strain B3AR132 are shown after 3 days of incubation in SRM medium supplemented with 0.1 to 1,000 μM of arbutin (●). Each value is the mean of three trials conducted on separate occasions; each trial consisted of duplicate cultures. Vertical bars indicate the standard error of the mean.

grown to the stationary phase. Populations of B3AR132 during the 5-day incubation period increased from an initial level of ~1 × 10^6 CFU/ml to 7 × 10^7 CFU/ml by day 2, a time when bacterial growth ceased but β-galactosidase activity was only about one-half of the maximum observed at day 3. Consequently, subsequent experiments used the cells grown for 3 days in SRM liquid medium as the inoculum and a 3-day incubation in routine tests of possible signal compounds for induction of β-galactosidase activity in SRM medium.

The influence of phenolic β-glucoside concentration on the induction of the *syrB*::*lacZ* fusion was tested to identify the concentration most favorable to expression of high activity. In addition to arbutin, esculin, and salicin, the study also included a fourth active phenolic compound, phenyl-β-D-glucopyranoside. Arbutin, phenyl-β-D-glucopyranoside, and salicin showed similar activity profiles at concentrations ranging from 0 to 1,000 μM, with peak β-galactosidase activities occurring at 100 μM (Fig. 2). However, the magnitude of *syrB* induction by esculin was only ~400 U, about 30% that of the other three phenolic β-glucosides. In all instances, no β-galactosidase activity was detected at concentrations of ≤0.1 μM, and only low activities of ≤30 U were observed at 1 μM. Activities at 1,000 μM declined by about 20 to 30% compared with peak activities at 100 μM. The largest change was a decrease of ~460 U for cultures supplemented with arbutin.

At arbutin concentrations ranging from 10 to 1,000 μM, a negative relationship was observed between *syrB* induction and viable cell numbers of B3AR132 (Fig. 2). Cell numbers averaged ~2 × 10^8 CFU/ml at arbutin concentrations of 0 to 1 μM, conditions in which no appreciable *syrB* induction was detected. At 100 μM arbutin, however, viable cell numbers were at their lowest value of ~6 × 10^7 CFU/ml, coinciding with maximum expression of β-galactosidase activity. Similar changes in viable populations were observed for B3AR253 and B3A-R grown in the presence of arbutin; sharp decreases in viable cell numbers were observed at 100 μM (data not shown).

Specificity of induction of *syrB*. Once incubation conditions conducive to high induction of *syrB* by arbutin were established, a collection of 34 phenolic compounds (including the 18 phenolic compounds initially surveyed above at 100 μM concentrations) were tested for activity at 1, 10, and 100 μM. Five of the 34 phenolic compounds tested were active. All five active phenolic compounds were β-glucosides and showed maximum induction at 100 μM. Arbutin, phenyl-β-D-glucopyranoside, and salicin (Fig. 3A) induced high levels of ~1,200 U of β-galactosidase activity at 100 μM, whereas esculin and helicin (Fig. 3B) induced moderate levels of ~300 U at 100 μM (Table 1). They are all phenolic glucosides derived from a single benzene ring (e.g., arbutin) or a coumarin ring (e.g., esculin) (Fig. 3). Phenolic compounds lacking *syrB*-inducing activity included several glycosides (amygdalin, fraxin, naringin, phloridzin, rhapontin, rutin, and 4-methylumbelliferyl-β-D-glucopyranoside), flavonoids (apigenin and naringenin) that induce *nod* genes in *Rhizobium* species (38), and acetosyringone, which induces *vir* genes in *A. tumefaciens* (35).

The relationship of chemical structure to signal activity was examined by testing substituted derivatives and components of active phenolic β-glucosides. The importance of β-glucosidic linkages in arbutin, phenyl-β-D-glucopyranoside, salicin, and esculin for signal activity were evaluated by testing their respective phenolic aglucone moieties (hydroquinone, saligenin, phenol, and esculetin). All four phenolic aglucones were inactive based on the recovery of <20 U of β-galactosidase activity, suggesting that the glucosidic linkage was crucial to activity.

To determine whether substitution of another sugar, such as galactose for glucose in the β-glucosidic linkage, affected activity, we compared phenyl-β-D-glucopyranoside with

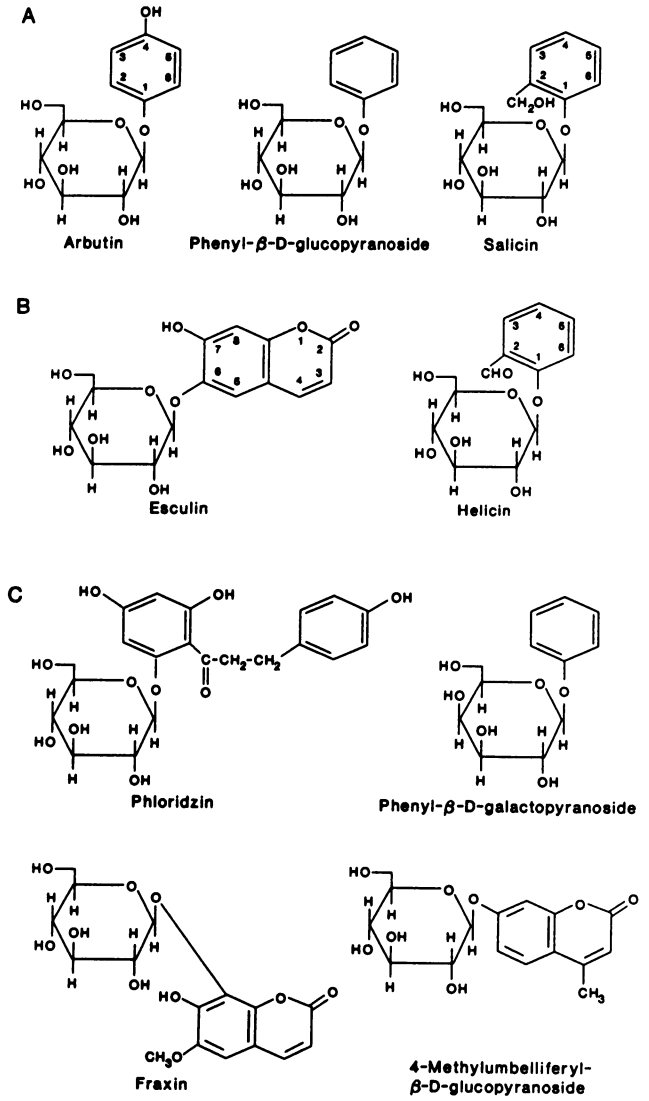

FIG. 3. Structures of phenolic β-glycosides exhibiting high (arbutin, phenyl-β-D-glucopyranoside, and salicin), intermediate (esculin and helicin), and no (phloridzin, phenyl-β-D-galactopyranoside, fraxin, and 4-methylumbelliferyl-β-D-glucopyranoside) induction activity of the *syrB*::*lacZ* fusion in strain B3AR132 of *P. syringae* pv. syringae.

phenyl-β-D-galactopyranoside for *syrB*-inducing activity (structures shown in Fig. 3A and C, respectively). Although phenyl-β-D-glucopyranoside consistently induced over 1,200 U of β-galactosidase activity in B3AR132 at a concentration of 100 μM, only 15 U of activity were obtained for phenyl-β-D-galactopyranoside.

Modifications of the benzene or coumarin rings were also evaluated for effects on signal activity. Substitution of the benzene ring of phenyl-β-D-glucopyranoside by either a hydroxyl group at position 4 (i.e., arbutin) or a hydroxymethyl group at position 2 (i.e., salicin) did not affect activity, but substitution of an aldehyde at position 2 (i.e., helicin) reduced activity by ~80% (Fig. 3; Table 1). Phloridzin, a β-glucoside found in apple (*Malus* spp.) tissue (22), showed no *syrB*-inducing activity. Phloridzin contains two hydroxyl-substituted benzene rings linked by a 4-hydroxyphenyl-1-propanone group (Fig. 3C). If a coumarin ring was

TABLE 1. Effect of plant phenolic β-glucosides on induction of a *syrB*::*lacZ* fusion in strain B3AR132 of *P. syringae* pv. syringae

Phenolic compound added[a]	Mean β-galactosidase activity (U) ± SE[b]
None	11 ± 1
Arbutin	1,243 ± 119
Phenyl-β-D-glucopyranoside	1,204 ± 100
Salicin	1,148 ± 78
Esculin	393 ± 44
Helicin	249 ± 15

[a] All phenolic β-glycosides were tested in SRM medium at 100 μM.
[b] Activities are the mean of three independent assays followed by the standard error. Each assay consisted of duplicate samples from two cultures.

substituted for the benzene ring, the *syrB*-inducing activity was reduced to ~400 U for esculin (Table 1). Even though fraxin and 4-methylumbelliferyl-β-D-glucopyranoside are derivatives of coumarin β-glucosides (Fig. 3C), they did not exhibit detectable signal activity.

Effect of sugars on arbutin-mediated induction of *syrB*. Eleven monosaccharides (L-arabinose, D-fructose, D-fucose, L-fucose, D-galactose, D-glucose, D-mannose, L-mannose, L-rhamnose, D-ribose, and D-xylose), five disaccharides (D-cellobiose, α-lactose, lactulose, maltose, and sucrose), one trisaccharide (D-raffinose), three sugar alcohols (*myo*-inositol, D-mannitol, and D-sorbitol), and two organic acids (pyruvate and citrate) were tested for effects on arbutin-mediated induction of *syrB* in SRM medium. The signal activity of arbutin at 10 μM was enhanced by approximately two- to fivefold by the addition (0.1%) of D-fructose, D-fucose, D-mannose, sucrose, or lactulose (Table 2). An arbutin concentration of 10 μM was used in these tests because the induction of *syrB* in the presence of fructose was more intense than at arbutin concentrations of either 1 or 100 μM (Fig. 4). The active sugars were divided into two categories based on ability to induce *syrB* when arbutin was absent in SRM medium. The first category was composed of D-fructose and sucrose, which induced *syrB* expression of ~100 U of β-galactosidase activity in SRM medium, and the second was composed of D-mannose, D-fucose, and lactulose, which did not show intrinsic *syrB*-inducing activities. Addition of fructose to cultures containing arbutin (10 μM) yielded the highest activity of ~1,200 U of β-galactosidase, which was more than five times the activity of the culture

TABLE 2. Effect of arbutin plus sugars on induction of a *syrB*::*lacZ* fusion in strain B3AR132 of *P. syringae* pv. syringae

Test sugar[a]	Mean β-galactosidase activity (U) ± SE[b]		
	Sugar alone	Sugar + arbutin	Fold increase
None	12 ± 3	215 ± 30	1.0
D-Fructose	94 ± 6	1,167 ± 368	5.4
Sucrose	118 ± 20	992 ± 29	4.6
D-Mannose	16 ± 3	838 ± 117	3.9
Lactulose	16 ± 4	564 ± 42	2.6
D-Fucose	13 ± 2	391 ± 134	1.8
D-Galactose[c]	14 ± 2	225 ± 13	1.0

[a] All sugars were tested in SRM medium at a final concentration of 0.1%.
[b] Activities are the mean of three independent assays followed by the standard error. Each assay consisted of duplicate samples from two cultures. Arbutin was added to SRM medium at a concentration of 10 μM.
[c] The following sugars showed background levels of β-galactosidase activity similar to that of D-galactose: L-arabinose, D-cellobiose, L-fucose, D-glucose, α-lactose, maltose, L-mannose, D-raffinose, L-rhamnose, D-ribose, and D-xylose.

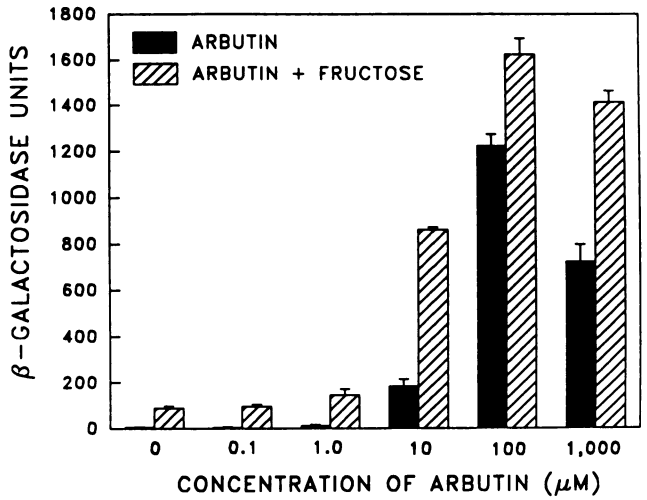

FIG. 4. Effect of D-fructose (0.1%) on induction of the *syrB*::*lacZ* fusion (as β-galactosidase units) in strain B3AR132 of *P. syringae* pv. syringae grown in SRM liquid medium supplemented with 0.1 to 1,000 μM of arbutin. Control cultures for each arbutin concentration were not supplemented with D-fructose. Each value is the mean of two trials conducted on separate occasions; each trial consisted of duplicate cultures. Vertical bars indicate the standard error of the mean.

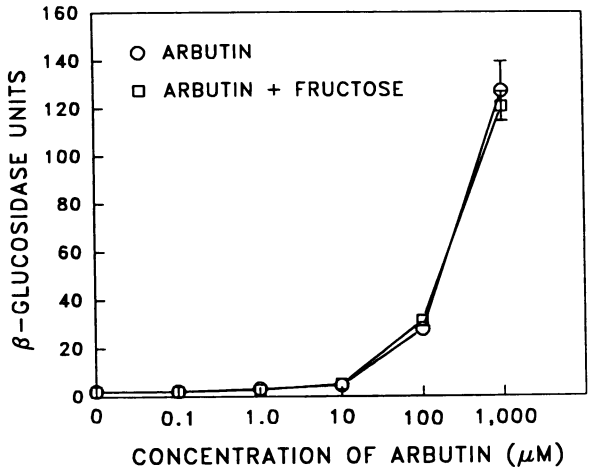

FIG. 5. Effect of arbutin concentration on expression of β-glucosidase activity by strain B3AR132 of *P. syringae* pv. syringae grown for 3 days in SRM liquid medium and SRM medium containing 0.1% fructose. The arbutin concentrations tested ranged from 0.1 to 1,000 μM. Each value is the mean of three trials conducted on separate occasions; each trial consisted of duplicate cultures. Vertical bars indicate the standard error of the mean.

containing arbutin alone. None of the other 12 sugars, three sugar alcohols, or two organic acids tested enhanced arbutin-mediated induction of *syrB*.

The above results suggested that sugars such as fructose increased the sensitivity of *syrB* expression to the arbutin signal. The effect of arbutin concentrations ranging from 0 to 1,000 μM on *syrB* induction was tested with or without fructose (0.1%) (Fig. 4). In the absence of fructose, appreciable induction of *syrB* occurred only at arbutin concentrations of 10 μM or higher. However, 1 μM arbutin plus fructose yielded 144 U of β-galactosidase activity, which was ~50 U higher than the intrinsic activity with fructose alone (95 U). At 10 μM arbutin, the enhancement effect of fructose on arbutin signal activity was the most notable, with more than a fourfold enhancement. Nearly 900 U of β-galactosidase activity were obtained in cultures containing fructose compared with ~200 U for cultures lacking fructose. At arbutin concentrations of 100 and 1,000 μM, the differential response to the addition of fructose was less striking, with only a 1.3- and 2-fold enhancement, respectively. In addition, the effect of fructose concentration on *syrB* induction by arbutin (10 μM) was tested at concentrations ranging from 0.0001 to 0.5%. The minimum concentration at which fructose enhanced arbutin-*syrB* induction was 0.001% (data not shown). Fructose also similarly enhanced the signal activities of other active phenolic β-glucosides (data not shown). The signal activities of phenyl-β-D-glucopyranoside, salicin, esculin, and helicin were all increased by greater than twofold over levels obtained with cultures lacking fructose. Nevertheless, phenolic compounds that showed no signal activity alone remained inactive upon addition of fructose.

Strain B3AR132 produces a β-glucosidase that can hydrolyze arbutin to yield glucose and the inactive phenolic, hydroquinone. To test whether fructose enhanced the arbutin-mediated *syrB* induction by suppressing the β-glucosidase activity of strain B3AR132, we measured β-glucosidase activity in SRM cultures containing either arbutin or arbutin plus fructose (Fig. 5). High concentrations of arbutin were required for induction of β-glucosidase activity in strain B3AR132. At 100 and 1,000 μM concentrations of arbutin, 26 and 128 U of β-glucosidase activity, respectively, were detected. Fructose did not inhibit β-glucosidase activity. If fructose (0.1%) was added to SRM medium containing the above arbutin concentrations, nearly identical induction levels of β-glucosidase activity were observed.

Effect of hydroquinone and saligenin on arbutin-mediated induction of *syrB*. It was observed (Fig. 2) that arbutin had lower signal activity at 1,000 μM than at 100 μM, and there was no significant difference in cell viability at the two arbutin concentrations. Therefore, tests were conducted to determine whether hydroquinone, which would be released by cleavage of arbutin by β-glucosidase, was associated with the reduction in β-galactosidase activity. Saligenin, the aglucone product of salicin, also was tested for possible inhibitory effects. Both hydroquinone and saligenin were tested at 10, 100, and 500 μM in SRM medium containing arbutin (100 μM) (Fig. 6). At concentrations of 100 and 500 μM, hydroquinone and saligenin significantly inhibited *syrB* induction by 20 to 50%. Moreover, the inhibition of β-galactosidase activity by hydroquinone was about twice that of saligenin when compared at equivalent concentrations. Neither hydroquinone nor saligenin inhibited bacterial viability at the concentrations tested.

Induction by plant signals of syringomycin production. Three strains of *P. syringae* pv. syringae (B301D-R, B3A-R, and HS191) were tested for effects of arbutin and fructose on syringomycin production under defined cultural conditions (Table 3). All three strains produced over 500 U of syringomycin per ml in PDB, whereas in SRM medium, only strain B301D-R produced the phytotoxin. Arbutin, fructose, or arbutin plus fructose had no effect on syringomycin production by strain B301D-R, which yielded 1,024 U/ml in SRM medium supplemented with arbutin, fructose, or arbutin plus fructose. In SRM medium with 100 μM of arbutin, strain HS191 produced 16 U of syringomycin per ml, but strain B3A-R produced no syringomycin despite the ability of

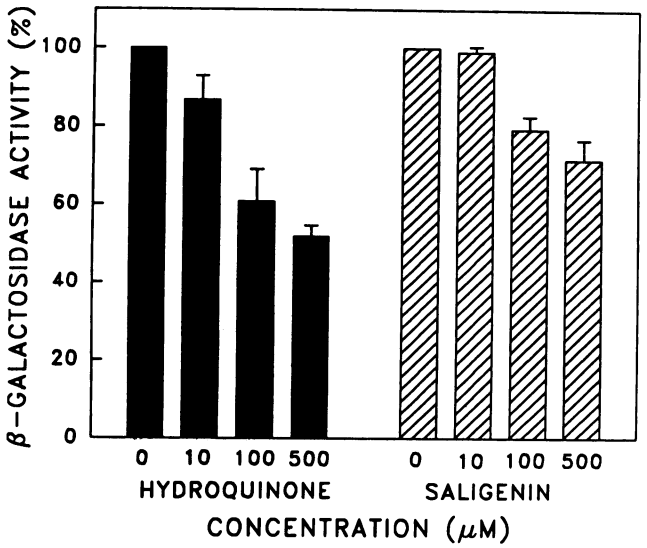

FIG. 6. Inhibitory effects of hydroquinone and saligenin on arbutin-mediated induction of the *syrB*::*lacZ* fusion in strain B3AR132 of *P. syringae* pv. syringae. Strain B3AR132 was grown for 3 days in SRM liquid medium supplemented with 100 μM arbutin and 10, 100, or 500 μM of hydroquinone or saligenin. Percent activities for the hydroquinone and saligenin treatments were determined relative to that of strain B3AR132 grown in SRM medium supplemented with 100 μM arbutin. Each value is the mean of three trials conducted on separate occasions; each trial consisted of duplicate cultures. Vertical bars indicate the standard error of the mean.

arbutin to induce the *syrB*::*lacZ* fusion in the derivative strain, B3AR132, in SRM medium (Fig. 1). However, the addition of fructose alone induced strain B3A-R to produce 32 U/ml. Furthermore, if both arbutin and fructose were added to SRM medium, syringomycin production by strains B3A-R and HS191 was increased substantially to 256 and 64 U/ml, respectively. Arbutin itself did not inhibit growth of *G. candidum*.

DISCUSSION

The induction in *P. syringae* pv. syringae of the *syrB* gene and syringomycin production by specific plant metabolites demonstrates that a gene required for virulence in a bacterium other than a member of the family *Rhizobiaceae* is modulated by the perception of signals in the plant environment. It is not surprising that a sensory mechanism is linked to virulence in a phytopathogen such as *P. syringae* pv. syringae, because it ensures that the bacterium is tightly attuned to a dynamic plant environment governing disease development. The efficient activation of the *syrB*::*lacZ* fusion in strain B3AR132 by phenolic compounds and sugars common to plant tissues indicates that induction of syringomycin production resembles the process of *vir* gene activation in *A. tumefaciens* (1, 5, 31). Nevertheless, there are notable differences in the types of plant metabolites displaying activity, and this may be related to differences in how the two bacteria parasitize plants and the chemical composition of the tissues invaded. *P. syringae* pv. syringae responds to phenolic β-glucosides and sugars, such as sucrose and fructose, that are common constituents of leaf and stem tissues attacked by the bacterium. In contrast, *A. tumefaciens* invades wounds and, accordingly, responds to phenolic and sugar signals that accumulate at wound sites (1).

TABLE 3. Effect of arbutin and fructose on syringomycin production by three strains of *P. syringae* pv. syringae

Medium[a]	Syringomycin production (U/ml)[b] by strain:		
	B301D-R	B3A-R	HS191
PDB	1,024	1,024	516
SRM	1,024	0	0
SRM + arbutin	1,024	0	16
SRM + fructose	1,024	32	16
SRM + arbutin + fructose	1,024	256	64

[a] Arbutin was added to SRM medium to a final concentration of 100 μM, and fructose was added to SRM medium to a final concentration of 0.1%.
[b] Average of two 50-ml cultures incubated for 5 days.

The induction of *syrB* in *P. syringae* pv. syringae by phenolic glucosides signifies a new class of phenolic compounds that function as signal molecules in plant-microbe interactions. A glucosylated benzene ring, as displayed by phenyl-β-D-glucopyranoside (Fig. 3C), is the fundamental structural requirement necessary for maximum *syrB*-inducing activity. All activity is lost if the glucosidic linkage is cleaved or if D-galactose is substituted for D-glucose. This indicates that the sugar moiety has a determinative effect on signal activity. Nevertheless, activity is not affected by substitution of the benzene ring of phenyl-β-D-glucopyranoside with either a hydroxyl or hydroxymethyl group as found in arbutin and salicin, respectively. The lower activity displayed by helicin suggests that certain chemical substitutions, such as an aldehyde group, are less tolerated. We also observed that esculin, a glucoside containing a coumarin ring, exhibits moderate signal activity, whereas the related coumarin glucosides, fraxin and 4-methylumbelliferyl-β-D-glucopyranoside, were inactive. Although this reinforces the chemical specificity displayed by the *syrB*-inducing signals, many more species of phenolic β-glucosides surely function as signal molecules among the wide range of plant species attacked by *P. syringae* pv. syringae (3). Phenolic compounds are generally accumulated and stored in plants as glycosides rather than as aglycones to effectively enhance water solubility and decrease chemical reactivity (14). Hence, phenolic glucosides are more accessible during invasion by a bacterium such as *P. syringae* pv. syringae.

The phenolic β-glucosides exhibiting *syrB*-inducing activity are found in the leaves, bark, and flowers of many plant species that are within the host range of *P. syringae* pv. syringae (3). Arbutin occurs in several species distributed among 10 dicot families, including *Rosaceae*, *Leguminosae*, and *Saxifragaceae*, and one monocot family, *Liliaceae* (7, 22, 28). Salicin, esculin, and helicin are also found in diverse plant families including *Caprifoliaceae* and *Salicaceae* (salicin), *Compositae* and *Oleaceae* (esculin), and *Rosaceae* (helicin) (4, 22, 28). The phenolic glycosides can occur in high concentrations in plant tissues, although the bulk is compartmentalized in vacuoles (14). For example, pear (*Pyrus communis*) leaves are reported (22) to contain 3 to 5% arbutin, which is equivalent to ~150 mM. Because the *syrB*::*lacZ* fusion in strain B3AR132 showed substantial induction at arbutin concentrations of ≥10 μM, it appears that the arbutin signal would be present in sufficient quantity to induce syringomycin production by *P. syringae* pv. syringae as it invaded pear tissues. The intensity of the induction would be further enhanced by the presence of soluble carbohydrates such as sucrose.

The phenolic signal compounds that induce the *syrB* gene

of *P. syringae* pv. syringae are chemically distinct from those that activate the *vir* genes of *A. tumefaciens* (20, 33, 35) and the *nod* genes of *Rhizobium* species (8, 29, 38). First, intact phenolic glucosides exhibit activity in *P. syringae* pv. syringae but not in *A. tumefaciens* and *Rhizobium* species. Coniferin, a phenylpropanoid glucoside, was isolated from a pinaceous gymnosperm by Morris and Morris (26) and shown to induce β-galactosidase activity from a *virE-lacZ* fusion in *A. tumefaciens* at a level comparable to that induced by acetosyringone. However, virulence gene induction was correlated with β-glucosidase activity that converted coniferin to the active aglucone, coniferyl alcohol. Although flavonoids are primarily found as *O*-glycosides in plants, glycosylation effectively eliminates the ability of the flavonoid signal molecule to induce nodulation genes in *Rhizobium* species (8). For example, glycosylation of the hydroxyl group of naringenin at position 7 to yield naringin substantially reduced or eliminated the ability to induce *nod* genes (8). The report (9) that apigenin-7-*O*-glucoside is a *nod* gene inducer was later attributed to contamination by a low level of the aglucone apigenin, which is a strong inducer (8). Second, an acetophenone inducer of *vir* genes (acetosyringone [35]) and flavonoid inducers of *nod* genes (apigenin and naringenin [38]) did not activate the *syrB* gene. Ankenbauer and Nester (1), moreover, reported that salicin was not an inducer of *vir* genes. Consequently, the perception and transduction of the phenolic glucoside signal to the transcriptional apparatus of *syrB* in *P. syringae* pv. syringae may occur via a sensory mechanism that only superficially resembles those observed in *A. tumefaciens* and *Rhizobium* species.

The enhancement of phenolic glucoside-mediated induction of *syrB* by specific sugars common to plant tissues is an integral part of the signal transduction process. The mechanism that enables sugars to augment the sensitivity of *P. syringae* pv. syringae to the phenolic signal is unclear, but it may resemble the ChvE sensory system of *A. tumefaciens* (5). Comparisons of sugar specificities, however, reveal important differences between the two plant-microbe systems. Sucrose and D-fructose, which are unable to enhance the induction of *vir* genes (1, 5, 31), were the most active in enhancing induction of *syrB*; conversely, no activity was observed for several *vir*-inducing sugars, including D-galactose, D-xylose, and L-arabinose (1). Of the sugars tested, only D-mannose and D-fucose were active in both systems. Furthermore, sucrose and D-fructose induce low levels of ~100 β-galactosidase units of *syrB*::*lacZ* activity in the absence of the phenolic inducer, whereas *vir* gene induction cannot occur unless a phenolic signal such as acetosyringone is present (1, 5). Although this precludes the involvement of a glucose-galactose-binding protein in *P. syringae* pv. syringae similar to ChvE in the periplasm of *A. tumefaciens* (5), one can speculate that a sugar-binding protein(s) responsible for uptake of the sugars active in *P. syringae* pv. syringae is necessary for efficient induction of genes required for syringomycin production.

Fructose appears to have the optimal monosaccharide structure for *syrB* induction based on a 5.4-fold enhancement of the signal activity of arbutin. Because sucrose and lactulose are fructose-containing disaccharides linked to inactive monosaccharides (glucose and galactose, respectively), one can speculate that the fructose moiety is crucial to the activity of these disaccharides. Although the mechanism by which fructose enhances the induction of *syrB* will require further study, fructose does not enhance induction by stimulating bacterial growth or by suppressing β-glucosidase activity.

Sucrose, D-fructose, and D-mannose are among the principal sugars found in plants (23). Analysis of cherry tissues, for example, showed that sucrose and fructose, respectively, composed as much as 3 and 1% of the dry weight of tissues sampled (16). Such concentrations are much higher than the threshold concentrations needed to cause significant induction of *syrB* based on our observation that fructose was active at concentrations as low as 0.001% (10 ppm) in the presence of arbutin.

One curious observation is that *P. syringae* pv. syringae produces a β-glucosidase that cleaves the β-glucosidic linkage of phenolic signal molecules to yield the inactive aglucone moiety. Although the aglucones hydroquinone and saligenin did not affect cell viability even at high concentrations, they did inhibit expression of *syrB*. This may be due to competition between the active phenolic glucoside and the aglucone for a binding site to effectively modulate expression of the *syrB* gene. Nevertheless, the enzymatic release of the aglucone would appear to be of little consequence to *syrB* induction in a plant host since high levels of β-glucosidase activity were obtained only if extremely high substrate concentrations of ≥100 μM were available (Fig. 5). Furthermore, β-glycosides are not nutritionally utilized by *P. syringae* pv. syringae based on observations by Joubert et al. (15) that arbutin and salicin do not support in vitro growth of *P. syringae* pv. syringae. In contrast to *Escherichia coli*, the β-glucosidase found in phytopathogenic fluorescent pseudomonads is not a phospho-β-glucosidase (15).

Our discovery that plant signal molecules are necessary for syringomycin production by strains B3A-R and HS191 of *P. syringae* pv. syringae may help explain the historical observation that many strains produce the toxin only in culture media supplemented with a crude plant extract (24). A notable exception is strain B301D-R, which produces maximum quantities of the toxin in SRM medium lacking both a sugar and a phenolic glucoside displaying signal activity. But such strains of *P. syringae* pv. syringae are infrequently encountered, and B3A-R seems to display a more typical regulatory control mechanism for syringomycin biosynthesis. It remains to be determined, however, why arbutin, in the absence of a sugar signal such as fructose, induces the *syrB*::*lacZ* fusion in strain B3AR132 but not syringomycin production in parental strain B3A-R. In an earlier study (24), we observed that expression of the *syrB*::*lacZ* fusion during phytopathogenesis was stronger and more rapid in a B3A-R background (i.e., homogenote strain B3AR132) than in B301D-R (i.e., homogenote strain BR132); after only 24 h postinoculation, the *syrB*::*lacZ* fusion in B3AR132 expressed more than four times the β-galactosidase activity of BR132. This further suggests that strains such as B3A-R are favored by a native ability to respond quickly and strongly to the dynamic plant environmental conditions conducive to infection.

The *syrB*::*lacZ* fusion proved to be a powerful tool for monitoring expression of a virulence gene of *P. syringae* pv. syringae in response to plant signals. Consequently, the study established that phenolic glycosides in concert with common plant sugars activate the *syrB* gene as well as the whole syringomycin biosynthetic pathway in at least some strains of the bacterium. The number of *syr* genes transcriptionally activated in direct response to chemical stimuli of plant origin is unknown, but the apparent complexity of the syringomycin biosynthetic apparatus suggests that several *syr* operons compose the stimulon. One can also envision that other virulence genes of *P. syringae* pv. syringae besides those involved in toxin production are likewise

controlled by the same signal transduction process. Studies of the molecular processes responsible for perception of plant signal molecules, such as arbutin, and their transmission to the transcriptional apparatus of *syrB* offer fresh opportunities for defining events critical to the plant-pathogen interaction.

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