Plant Physiol. (1995) 107: 603–612

Analysis of Sweet Cherry (*Prunus avium* L.) Leaves for Plant Signal Molecules That Activate the *syrB* Gene Required for Synthesis of the Phytotoxin, Syringomycin, by *Pseudomonas syringae* pv *syringae*[1]

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An important aspect of the interaction of *Pseudomonas syringae* pv *syringae* with plant hosts is the perception of plant signal molecules that regulate expression of genes, such as *syrB*, required for synthesis of the phytotoxin, syringomycin. In this study, the leaves of sweet cherry (*Prunus avium* L.) were analyzed to determine the nature of the *syrB*-inducing activity associated with tissues of a susceptible host. Crude leaf extracts yielded high amounts of total signal activity of more than 12,000 units g^{-1} (fresh weight) based on activation of a *syrB-lacZ* fusion in strain B3AR132. The signal activity was fractionated by C_{18} reversed-phase high-performance liquid chromatography and found to be composed of phenolic glycosides, which were resolved in three regions of the high-performance liquid chromatography profile, and sugars, which eluted with the void volume. Two flavonol glycosides, quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside, and a flavanone glucoside, dihydrowogonin 7-glucoside, were identified. The flavonoid glycosides displayed similar specific signal activities and were comparable in signal activity to arbutin, a phenyl β-glucoside, giving rise to between 120 and 160 units of β-galactosidase activity at 10 μM. Although D-fructose exhibits intrinsic low level *syrB*-inducing signal activity, D-fructose enhanced by about 10-fold the signal activities of the flavonoid glycosides at low concentrations (e.g. 10 μM). This demonstrates that flavonoid glycosides, which represent a new class of phenolic plant signals sensed by *P. s. syringae*, are in sufficient quantities in the leaves of *P. avium* to activate phytotoxin synthesis.

Bacteria commonly detect in the plant environment specific phenolic compounds that serve as signals in regulating expression of genes critical to phytopathogenesis (Peters and Verma, 1990). This was first observed for *Agrobacterium tumefaciens*, which senses in wounded tissues a structurally diverse group of plant phenolic compounds (Stachel et al., 1985b). Perception of phenolic signal molecules leads to transcriptional activation of virulence (*vir*) genes necessary for the transfer of T-DNA to host cells and formation of crown galls. The types of phenolic compounds reported to have signal activity include certain acetophenones (e.g. acetosyringone), monolignols, chalcones, and flavonoids (Spencer and Towers, 1988; Zerback et al., 1989; Duban et al., 1993). In the rhizobia, specific plant flavonoids were discovered to activate nodulation (*nod*) genes essential for establishment of symbiosis (Peters et al., 1986). Relatively low flavonoid concentrations ranging from 0.1 to 10 μM are necessary for activation of *nod* genes in *Rhizobium* (Firmin et al., 1986). In comparison, higher concentrations of phenolic inducers ranging from 10 to 500 μM are required for activation of *vir* genes in *Agrobacterium* (Melchers et al., 1989). This is ameliorated by a second class of *vir*-inducing compounds composed of simple pyranose sugars that markedly enhance by 60- to 200-fold the activities of low concentrations of phenolic signal molecules such as acetosyringone (Ankenbauer and Nester, 1990; Cangelosi et al., 1990; Shimoda et al., 1990).

It recently was established that phytotoxin production by *Pseudomonas syringae* pv *syringae* is modulated by the perception of specific plant metabolites (Mo and Gross, 1991b; Quigley and Gross, 1994). Certain phenolic β-glucosides, such as arbutin, serve as signals that induce production of syringomycin, a cyclic lipodepsinonapeptide toxin that causes necrotic symptoms in host plants (Segre et al., 1989; Takemoto, 1992). In addition, the *syrB* gene, which is conserved in toxigenic strains of *P. s. syringae* and is predicted to encode a synthetase (Quigley and Gross, 1994; J.-H. Zhang, N.B. Quigley, and D.C. Gross, unpublished data), is activated by phenolic signal compounds. This was measured as induction of β-galactosidase activity resulting from a transcriptional fusion between *syrB* and a promoterless *lac* operon as a reporter of gene activity (Mo and Gross, 1991a, 1991b). In a survey of 34 plant phenolic compounds for *syrB*-inducing activity, which included representative *vir*- or *nod*-inducing phenolics, only certain phe-

[1] This work was supported in part by grants 90–37262–5297 and 92–37303–7732 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture, Science and Education Administration. This is journal paper PPNS 0185 of the College of Agriculture and Home Economics Research Center, Washington State University, Pullman, WA.

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Abbreviation: SRM, syringomycin minimal.

nolic glucosides exhibited *syrB*-inducing activity (Mo and Gross, 1991b). The β-glucosidic linkages in arbutin, phenyl-β-D-glucopyranoside, salicin, and esculin were concluded to be crucial to activity based on a lack of activity for their respective phenolic aglycone derivatives. In addition, a few mono- and disaccharides common to plant tissues substantially enhanced the *syrB*-inducing activity of phenolic signals, and this was most noticeable at low concentrations of phenolic glucoside (i.e. 1 to 10 μM). Suc and D-Fru were the most active enhancers of phenolic signal activity and also exhibited intrinsic low-level signal activity yielding about 100 β-galactosidase units of *syrB-lacZ* activity in the absence of the phenolic inducer. The influence of signal-mediated induction of phytotoxin production by *P. s. syringae* was emphasized by the production of larger quantities of toxin by more than 90% of a diverse spectrum of toxigenic strains in the presence of arbutin and D-Fru (Quigley and Gross, 1994). Some strains produced toxin in vitro only in the presence of these signals. Because syringomycin production is an important element of virulence in *P. s. syringae* (Xu and Gross, 1988a, 1988b; Gross, 1991), this demonstrates that the detection of plant signal molecules can have a governing role in the establishment of disease.

P. s. syringae is an important pathogen of sweet cherry (*Prunus avium* L.), causing stem cankers and necrotic lesions on fruits and leaves (Cameron, 1962; Jones, 1971). Cherry tissues appear to contain plant signal molecules that are perceived by *P. s. syringae* based on evidence that the *syrB-lacZ* fusion is transcriptionally activated in an environment containing plant constituents (Mo and Gross, 1991a). First, inoculations of immature cherry fruits demonstrated that there is a rapid and strong expression of *syrB* in situ. For example, the *syrB-lacZ* fusion of strain B3AR132 expressed peak activity of about 425 β-galactosidase units after only 24 h of incubation. Second, addition of a water-soluble extract from cherry leaves to SRM medium converted it into a medium favorable for expression of the *syrB-lacZ* fusion in strain B3AR132. When SRM medium was not amended with the cherry leaf extract, strain B3AR132 failed to exhibit detectable β-galactosidase activity. This demonstrated that the cherry leaf extract contained a constituent or signal that was sensed by the bacterium, eventually leading to transcriptional activation of *syrB*. Furthermore, studies by Krzesinska et al. (1993) indicated that susceptibility of cherry genotypes to bacterial canker is correlated with signal activity. Extracts from the stems of 12 cherry genotypes were tested for *syrB*-inducing activity, and the genotypes most susceptible to bacterial canker contained higher ($P = 0.01$) signal activities than resistant genotypes. Consequently, both the quantity and quality of plant metabolites with signal activity may have an acute effect on disease development.

Despite evidence that specific plant metabolites have a vital role as signals in the interaction of *P. s. syringae* with plant hosts, little is known about the number, quantity, and chemical species of signals contained in host tissues. The objective of this study was to determine the nature of the *syrB*-inducing activity associated with cherry leaves. Although an earlier survey (Mo and Gross, 1991b) identified a few phenolic β-glucosides with signal activity, none of the active substances are reported to occur in *P. avium* tissues. Moreover, a lack of *syrB*-inducing activity for several phenolic glycosides, such as amygdalin and phloridzin, indicated that relatively few plant phenolic substances can serve as signals. In a preliminary report (Geibel et al., 1994), we described the chromatographic and spectroscopic (UV and NMR) characteristics of quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside from *P. avium* leaves. Here we report details of the fractionation of phenolic signal activity from cherry leaves into three regions by HPLC. We demonstrate that two flavonol glycosides (quercetin and kaempferol 3-rutinosyl-4′-glucosides) and a flavanone glucoside (dihydrowogonin 7-glucoside) exhibit *syrB*-inducing activity and that their signal activities are enhanced substantially by D-Fru. The specific signal activities of the flavonoid glycosides are reported, and this is discussed in relation to the host-pathogen interaction.

MATERIALS AND METHODS

Bacterial Strains, Media, and Chemicals

Pseudomonas syringae pv *syringae* strain B3A-R and its Tox^- derivatives B3AR132 and B3AR253 were described by Mo and Gross (1991a, 1991b). Strain B3AR132 carries an in-frame *syrB-lacZ* fusion, generated by Tn3HoHo1 mutagenesis (Stachel et al., 1985a), recombined into the chromosome of B3A-R by marker exchange, whereas strain B3AR253 carries a *syrB-lacZ* fusion oriented opposite to the direction of *syrB* transcription. β-Galactosidase activity was expressed by strain B3AR132 under conditions conducive to syringomycin production (Mo and Gross, 1991a); strain B3AR253 does not express β-galactosidase activity. Methods used for routine culture of strains were described by Mo and Gross (1991b).

SRM liquid medium (Gross, 1985) was used for assays of *syrB* induction. SRM medium contains 55.5 mM D-Glc, 19.1 mM L-His, 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). Complete SRM medium was used as the basal medium in all tests of fractions from cherry leaves for *syrB*-inducing activity.

Arbutin, a phenolic β-glucoside with *syrB*-inducing activity (Mo and Gross, 1991b), rutin (quercetin 3-rutinoside), quercetin, quercetin 3-rhamnoside, and kaempferol were purchased from Sigma. Arbutin was dissolved in water, whereas quercetin, kaempferol, rutin, and quercetin 3-rhamnoside, which are not highly soluble in water, were dissolved in DMSO. Stock solutions (100 mM) were filter sterilized and stored at −20°C before use. All sugars, including D-Fru and D-Man, were purchased from Sigma.

Assays of Fractions from Cherry Leaves for *syrB*-Inducing Activity

Methods for preparing inocula of strains B3AR132 and B3AR253 for use in routine tests of *syrB* induction were as

described by Mo and Gross (1991b). The method of Miller (1972), as modified by Stachel et al. (1985a), was used to measure β-galactosidase activity resulting from expression of the *syrB-lacZ* fusion in strain B3AR132. All bioassays were performed in SRM medium containing test compounds (cherry leaf fractions or known phenolic compounds); all final volumes were 5 mL. Typically, leaf extracts and fractions from chromatographs (paper or HPLC) were dissolved in deionized water, filter sterilized, and then added to SRM medium in volumes of 10 to 50 μL. Insoluble fractions, sterilized as described below, were air dried and added in 10-mg quantities to SRM medium. Arbutin, tested at a final concentration of 10 μM, was included in all trials as a control for monitoring levels of *syrB* induction to ensure uniformity among experiments. Background levels of β-galactosidase activity were determined from cultures of B3AR132 grown in SRM medium lacking signal molecules or from cultures of the noninducible *syrB-lacZ* mutant, B3AR253. Conditions for incubation of cultures and processing of cells for β-galactosidase activity were as described in detail by Mo and Gross (1991b). In routine assays, duplicate cultures were prepared for all fraction samples tested for signal activity; two samples from each culture were assayed for β-galactosidase activity. In assays of pure phenolic compounds, two trials were conducted on separate days with each trial consisting of duplicate cultures.

The effects of D-Fru, D-Man, Suc, and other sugars (Mo and Gross, 1991b) on the activities of phenolic signal molecules were measured at a final concentration of 0.1% (w/v) in SRM medium. Quercetin, quercetin 3-rhamnoside, rutin, and kaempferol were tested for *syrB*-inducing activity at final concentrations of 10 and 100 μM, which were added as 5 μL of stock solution (dissolved in DMSO) to SRM medium. The 5-μL volumes of DMSO added to SRM medium were previously shown to have no effect on *syrB* induction (Mo and Gross, 1991b).

Extraction of Signal Molecules from Cherry Leaves

Cherry (*Prunus avium* L.) cv Bing, grown in an approximately 25-year-old orchard at the Irrigated Agriculture Research and Extension Center, Prosser, WA, was used as the source of leaves for extraction of signal molecules. Leaves were harvested in early May about 4 weeks after bloom. Prior to storage at −80°C, leaves were rinsed in running tap water, blotted dry with paper towels, and frozen in liquid nitrogen.

In initial experiments, leaves (100 g) were homogenized in deionized water, methanol (25, 50, 75, and 100%), or chloroform at a 1:3 ratio (w/v) to compare the relative recoveries of total signal activities. The homogenates were centrifuged (10,000*g*, 10 min), and the supernatants were reduced to dryness by flash evaporation. The dried extract was dissolved in 8 mL of water and filter sterilized; appropriate dilutions were tested in 50-μL aliquants for *syrB*-inducing activity.

Because the aqueous leaf extract yielded the highest total signal activity, it was further purified by extraction with polar solvents following the three steps summarized in Figure 1. After cherry leaves (100 g) were homogenized (i.e. step A) in deionized water at a 1:3 ratio (w/v), the homogenate was centrifuged (10,000*g*, 10 min) to pellet insoluble plant material. A 1-mL sample of the supernatant was collected for bioassay, filter sterilized, and stored at −80°C prior to bioassay. The pellet was sterilized by treatment with 70% ethanol and air dried. In step B, the supernatant was adjusted to a final concentration of 75% (v/v) acetone, stirred vigorously for 10 min, and centrifuged (10,000*g*, 10 min) to pellet insoluble material. A 4-mL sample of the supernatant was collected for bioassay; it was reduced to dryness by flash evaporation, redissolved in 1 mL of deionized water, filter sterilized, and stored at −80°C prior to bioassay. In step C, the bulk of the supernatant was reduced to dryness by flash evaporation and then extracted with 95% ethanol at 78°C for 20 min. After centrifugation (10,000*g*, 10 min), the ethanol-soluble fraction was reduced to dryness by flash evaporation, redissolved in 10 mL of deionized water, and filter sterilized. The pellets obtained in steps B and C were air dried, redissolved in 1 mL of deionized water, filter sterilized, and stored at −80°C prior to bioassay. The recoveries of signal activities were determined based on the dry and fresh weights of cherry leaf tissue.

HPLC Analysis of Signal Molecules

The crude cherry leaf extract was analyzed by HPLC for fractions containing signal activity. A 200-μL sample of the crude extract (Fig. 1) was injected into a Waters HPLC system (Millipore, Milford, MA) equipped with an Altex Ultrasphere-ODS column (250 × 10 mm, semipreparative C_{18} reversed-phase column; Beckman Instruments, Fullerton, CA). The column was eluted for 60 min at a flow rate

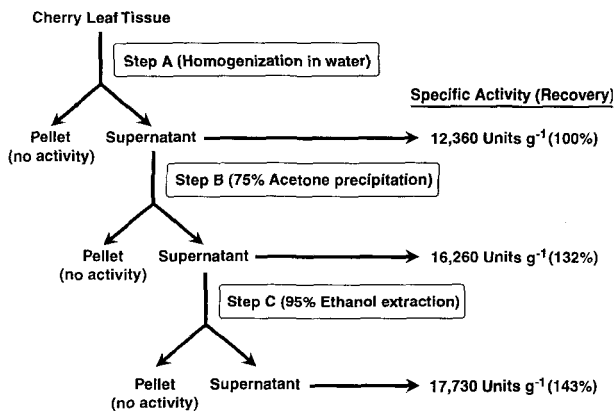

Figure 1. Recoveries of signal activities from cherry leaves at steps A, B, and C of the extraction procedure (see "Materials and Methods" for a description of the methodology used at each step). Specific activities of plant signals were measured as β-galactosidase units induced per g (fresh weight) of leaf tissue and were based on induction of the *syrB-lacZ* fusion in strain B3AR132 of *P. s. syringae*. Activities are the means of two independent trials conducted on separate occasions; each assay consisted of duplicate samples from two cultures.

of 2 mL min^{-1} with an initial equilibration for 2 min in 0.1% TFA, followed by a 0 to 60% methanol gradient in 0.1% TFA. The compounds were monitored at A_{214}, A_{254}, A_{266}, and A_{342} using a Waters 990 photodiode array detector. The eluent fractions were collected every minute, dried by lyophilization, and redissolved in 200 μL of water. After filter sterilization, a 10-μL sample from each fraction was assayed as described above for *syrB*-inducing activity.

Large-Scale Purification of Phenolic Signal Molecules

Initial HPLC separations of crude extracts identified a poorly resolved region at fractions 47 to 49, called region A (Fig. 2), with signal activity. Consequently, preparative paper chromatography of the crude ethanol extract from cherry leaves was used for large-scale purification. Paper chromatograms were developed for about 6 h in a butanol:ethanol:water (4:1:2.2) solvent system (Markham, 1982) and air dried. Prior analysis determined that the phenolic substances associated with region A were visible at A_{302} as a dark gray band with an R_F value of 0.35; more than half of the signal activity of the crude extract originally applied to the chromatogram was associated with this dark gray band. The band was excised from the paper and extracted overnight with deionized water (approximately 10 mL per band). The aqueous extract was reduced to dryness by flash evaporation, redissolved in 300 μL of deionized water, and then further purified by HPLC. HPLC peak fractions showing appreciable activity were individually rechromatographed by HPLC and examined for purity by a Waters 911 program.

To obtain more of the phenolic signal molecules in region A (Fig. 2), methanolic leaf extracts of *P. avium* cv Sam were used. For purification, a repeated combination of column chromatography (400 × 25 mm, Polyamid SC-6, 0.05–0.16 mm, solvent water) and preparative HPLC (250 × 20 mm, C_{18} Polygosil 5 μm, methanol gradient) was necessary. Larger amounts of dihydrowogonin 7-glucoside were obtained by isolation from cherry bark (Geibel et al., 1990).

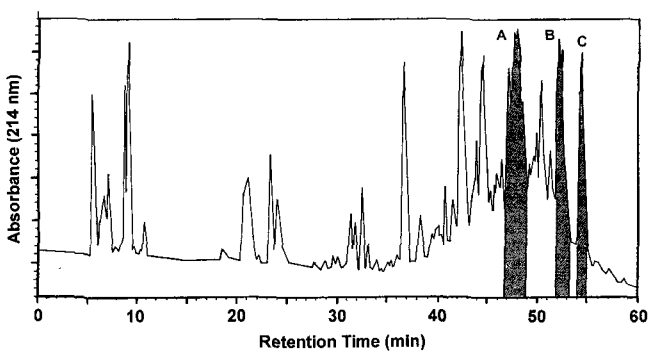

Figure 2. Fractionation by HPLC of a crude cherry leaf extract and identification of peak regions (A_{214}) having signal activity. The crude extract, prepared as shown in Figure 1, was fractionated on a reversed-phase C_{18} column with a 0 to 60% methanol gradient in 0.1% TFA. Phenolic signal activities, as determined by induction of the *syrB-lacZ* fusion in strain B3AR132 of *P. s. syringae*, were observed in three peak regions (shaded regions labeled A, B, and C): region A, fractions 47 to 49 subsequently identified as a mixture of quercetin glycosides and kaempferol glycosides; region B, fractions 52 and 53; and region C, fractions 54 and 55 subsequently identified as dihydrowogonin 7-glucoside. Signal activity also was observed in fractions 6 and 7 (void volume), which contained Fru. None of the other peaks and fractions contained signal activity.

Identification of the Phenolic Signal Molecules

Techniques used for identification of region A (HPLC, TLC, enzymatic and acidic hydrolysis, UV spectroscopy, and NMR) were described by Geibel et al. (1994). In region C the phenolic signal molecule was identified by HPLC, UV spectroscopy, and TLC by comparison with an authentic sample of dihydrowogonin 7-glucoside from Treutter et al. (1985).

Identification of HPLC Fraction 7, Which Enhances Phenolic Signal Activity

HPLC fraction 7 enhanced the signal activity of the flavonoid glycosides isolated from cherry leaves, and thus was analyzed by TLC for the presence of sugars with enhancer activity. Samples from HPLC fraction 7 along with 14 sugar standards (i.e. L-Ara, D-cellobiose, D-Fru, L-Fuc, D-Gal, D-Glc, α-lactose, maltose, D-Man, D-raffinose, L-rhamnose, D-Rib, Suc, and D-Xyl prepared as 10% [w/v] stocks) were loaded (5 μL per spot) onto silica gel type 60A TLC plates (Whatman) and run in a butanol:ethanol:water (4:1:2.2) solvent system for 5 h (Markham, 1982). After air drying, TLC plates were sprayed with a sugar-detecting reagent (3% phenol and 5% sulfuric acid in 95% ethanol) and developed at 100°C for 10 min (Bruno and Svoronos, 1989). The R_F values of reactive spots were calculated at the center of each spot. TLC analysis of HPLC fraction 7 yielded a single spot with an R_F value of 0.27; this region was scraped from preparative TLC plates, extracted overnight with deionized water, and dried by lyophilization. The sample was redissolved in deionized water (200 μL) and filter sterilized prior to assay for ability to enhance the signal activities of arbutin and the compound purified as peak 2 from HPLC region A (subsequently identified as quercetin 3-rutinosyl-4′-glucoside).

RESULTS

Extraction of *syrB*-Inducing Signal Molecules from Cherry Leaves

An aqueous homogenate of cherry leaves yielded high amounts of total signal activity of more than 12,000 units g^{-1} (fresh weight) of β-galactosidase activity (Fig. 1); on a dry weight basis total signal activity was measured to be about 160,000 units g^{-1}. The signal activity was not extracted with chloroform, a nonpolar solvent. Extractions with 25, 50, 75, and 100% methanol yielded recoveries of 98, 44, 40 and 11%, respectively, of the signal activity of an aqueous extract. The signal activity was resistant to exposure to heat (e.g. 100°C, 10 min) and could pass through a dialysis membrane with a 1000 mol wt cutoff.

Extraction of the crude aqueous extract with 75% acetone increased the specific activity by about 32% (step B, Fig. 1); extractions with either 50 or 100% acetone yielded lower recoveries of signal activity. Subsequent treatment with 95% ethanol (step C, Fig. 1) yielded an additional 11% increase in the specific activity of the original crude extract; on a dry weight basis this represented a 3.7-fold increase in the specific activity as compared to total signal activity after step A. No appreciable signal activity was associated with the insoluble fractions that were pelleted by centrifugation.

HPLC Fractionation of Phenolic Signal Activity into Three Regions

The ethanol-soluble cherry leaf extract fractionated by C_{18} reversed-phase HPLC contained three reproducible *syrB*-inducing regions located at retention times between 47 and 55 min in a 60-min run (Fig. 2). Spectral analysis of all three peak regions indicated the presence of flavonoids (Markham, 1982) based on the occurrence of two absorption maxima that were monitored at A_{266} and A_{342}. Most of the *syrB*-inducing activity was associated with region A, which was composed of at least two peaks; region A accounted for approximately 42% of the recovered signal activity. Regions B and C also displayed significant signal activity as observed by the recoveries of 25 and 21%, respectively, of the recovered signal activity. In addition, about 12% of the recovered signal activity was eluted with the void volume between 6 and 7 min. Several other peaks, observed at A_{214} (Fig. 2), were resolved by HPLC of the cherry leaf extract, but they did not exhibit signal activity.

Because region A contained most of the signal activity extracted from cherry leaves, emphasis was placed on purifying and identifying the phenolic substances within region A. Preparative paper chromatography was used to concentrate and partially purify the signal molecules in region A prior to HPLC analysis. The crude ethanol-soluble fraction was resolved into eight bands detectable by UV illumination at A_{302}. The fourth band (R_F = 0.35) was dark gray and accounted for about 60% of the initial *syrB*-inducing activity applied to the chromatogram. The phenolic substances from this band were fractionated by C_{18} reversed-phase HPLC and shown to correspond with the phenolic signals identified as region A (Fig. 2). Unlike the HPLC profile of the ethanol-soluble fraction, however, signal regions B and C were not recovered. Region A was separated into three peaks, two major peaks and one minor peak with retention times ranging from 65 to 69 min in an 80-min run (Fig. 3A). Assays of *syrB* induction revealed two peaks of activity corresponding to fractions with retention times of 66 to 67 (peak 2) and 68 to 69 (peak 3), accounting for 8.2 and 6.8%, respectively, of the initial unfractionated signal activity (approximately 57,000 units total of β-galactosidase activity) added to the HPLC column (Fig. 3B). Total recovery of the peak-2 and -3 signal molecules was estimated to be about 4 mg/g (fresh weight) of leaf tissue. In addition, low signal activity (3% of the original activity) was detected in fractions 7 and 8, which

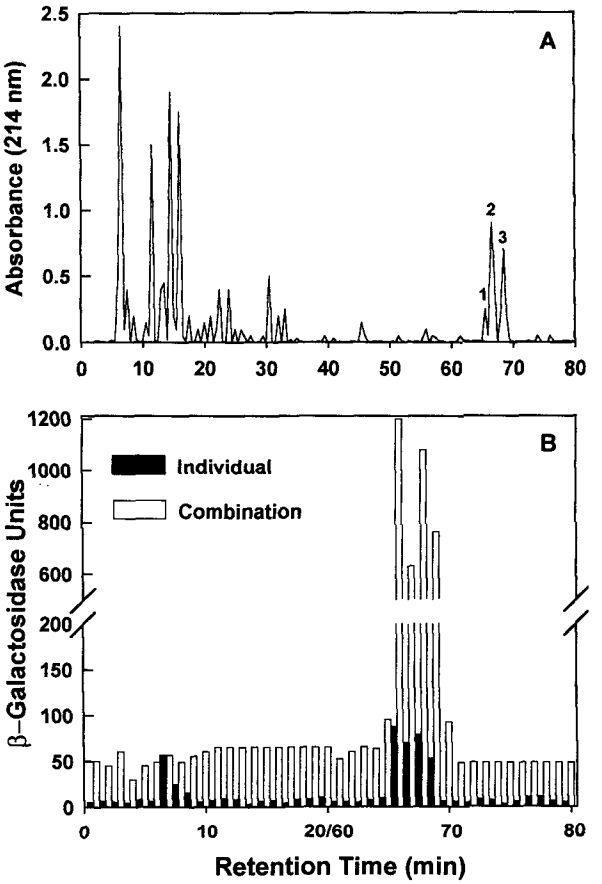

Figure 3. Separation by HPLC of phenolic glycosides from cherry leaves that are associated with the signal activity found in region A (shown in Fig. 2). Prior to fractionation on a reversed-phase C_{18} column (0–40% methanol gradient in 0.1% TFA), the crude extract, prepared as shown in Figure 1, was partially purified by preparative paper chromatography. A, Three peaks were resolved at A_{214} and subsequently identified as peak 1, a mixture of quercetin 3,4′-diglucoside and kaempferol 3,4′-diglucoside; peak 2, quercetin 3-rutinosyl-4′-glucoside; and peak 3, kaempferol 3-rutinosyl-4′-glucoside. B, Signal activities of HPLC fractions assayed, in 10-μL aliquants, either individually (solid bars) or in combination (open bars) with 10 μL of fraction 7. HPLC fraction 7 was collected as the void volume and subsequently was found to be composed primarily of Fru. Activities such as β-galactosidase units were determined by induction of the *syrB-lacZ* fusion in strain B3AR132 of *P. s. syringae*. No signal activity was detected between HPLC fractions 20 to 60.

contain nonphenolic compounds based on a lack of absorbance at A_{254}. A low specific activity of 75 β-galactosidase units mg^{-1} was measured for HPLC fraction 7. Thus, the recovery of signal activity from individual HPLC fractions totaled 18% of the original activity added to the HPLC column.

Pooling all HPLC fractions resulted in full recovery of signal activity, suggesting that phenolic signal activity was enhanced by a substance fractionated elsewhere in the HPLC profile. Pooling of HPLC fractions in various combinations identified fractions 7 to 8, eluting as the void volume, to contain enhancer activity; no other HPLC frac-

tions displayed enhancer activity. As observed in Figure 3B, HPLC fraction 7 caused about a 10-fold increase in the activities of peaks 2 and 3. Combined signal activities of peaks 2 and 3 in the presence of HPLC fraction 7 was estimated to total about 100,000 units, which was nearly twice the activity of the unfractionated sample.

HPLC Fraction 7 Is Composed of a Sugar That Enhances Phenolic Signal Activity

HPLC fraction 7 added at a concentration of 0.1% (w/v) enhanced the signal activity of arbutin by more than 5-fold. Such levels of enhanced *syrB* induction in the presence of HPLC fraction 7 were similar to the effects of a few sugars, including D-Fru and D-Man, on phenolic signal activity (Mo and Gross, 1991b). Separation of HPLC fraction 7 by TLC followed by treatment with the phenol-sulfuric acid reagent for sugars revealed a single dark greenish-blue spot with an R_F value of 0.27 (not shown). Compared to 14 sugar standards, only D-Fru and D-Man had similar R_F values and coloration. Assays for *syrB* induction showed that all enhancer activity separated by preparative TLC was associated with the greenish-blue area and that it had intrinsic but relatively low *syrB*-inducing activity.

The *syrB*-inducing activities of arbutin and quercetin 3-rutinosyl-4′-glucoside (i.e. peak 2 of region A) were similarly enhanced by the same set of sugars. For example, D-Fru caused a 10- to 12-fold increase in their activities when assayed at low concentrations (Table I). D-Man, which does not exhibit intrinsic *syrB*-inducing activity (Mo and Gross, 1991b), caused a 4-fold increase in the activities of both phenolic signal molecules (data not shown).

Identifications of Phenolic Compounds with Signal Activity

Region A

Peaks 2 and 3 of HPLC region A (Figs. 2 and 3) were identified, respectively, as quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside (Fig. 4). In methanol, peak 2 showed A_{max} at 256, 355 nm and a shoulder at 268 nm, and peak 3 showed A_{max} at 265, 350 nm

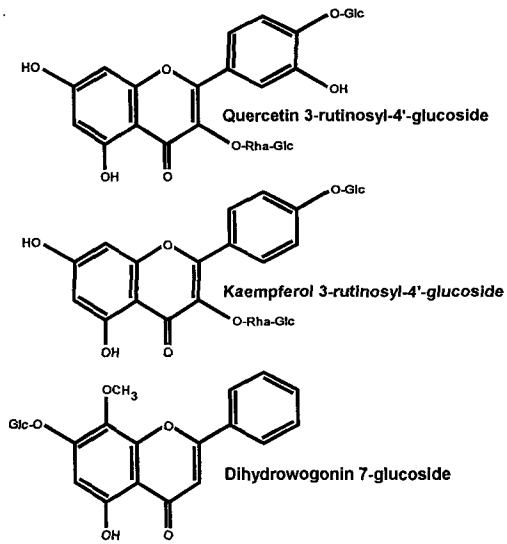

Figure 4. Structures of flavonoid glycosides from cherry (*P. avium* L.) leaves with signal activity, based on induction of the *syrB* gene of *P. s. syringae*.

and shoulders at 244, 315 nm, which are characteristic of flavonols (Markham, 1982). The assignment of these structures is based on the following analytical data: (a) the release of the aglycones quercetin (peak 2) and kaempferol (peak 3) by acidic hydrolysis, (b) the corresponding flavonol 3-rutinosides were released by hydrolysis with β-glucosidase and confirmed by comparison to authentic samples by HPLC and TLC, (c) confirmation by UV spectroscopy and TLC analysis that the hydroxyl groups at positions 3 and 4′ are both glycosylated and that the hydroxyls at positions 5 and 7 are free (Geibel et al., 1994), and (d) final confirmation by ^{1}H-NMR spectroscopy (Geibel et al., 1994).

Peak 1 (Fig. 3) was identified as a mixture of 3,4′-diglucosides of quercetin and kaempferol. In methanol, peak 1 had A_{max} at 256, 355 nm and a shoulder at 268 nm (Geibel et al., 1994). The structures were assigned based on (a)

Table I. *The syrB gene inducing activity of flavonoid glycosides isolated from sweet cherry leaves*

Assays in SRM medium measured β-galactosidase activity produced from an *syrB-lacZ* fusion in strain B3AR132 of *P. s. syringae*. SRM was supplemented with D-Fru to a final concentration of 0.1% in tests for enhancement of phenolic signal activity. Background β-galactosidase activities of 8 to 12 units were subtracted, and each value (± SE) is the mean of two trials conducted on separate occasions; duplicate cultures per trial were assayed after 3 d of incubation.

Phenolic Signal	Purity	Concentration	β-Galactosidase Activity		
			Without Fru	With Fru	Increase
	%	μM	units		fold
Quercetin 3-rutinosyl-4′-glucoside	98–99	10	143 (±41)	1381 (±85)	9.7
Kaempferol 3-rutinosyl-4′-glucoside	93–96	10	134 (±35)	1462 (±91)	10.9
Dihydrowogonin 7-glucoside	92	10	162 (±22)	1720 (±82)	10.6
Arbutin	Commercial	10	120 (±13)	1447 (±54)	12.1
None			0	101 (±5)	

HPLC retention times and R_F values in TLC suggestive of a diglycoside with glycosylation at positions 3 and 4′ (fluorescence), (b) ^{1}H-NMR spectroscopy that showed no rhamnose but two Glc molecules linked to hydroxyl groups at positions 3 and 4′ (Geibel et al., 1994), and (c) the occurrence of both quercetin and kaempferol based on ^{1}H-NMR signals of aromatic groups (Geibel et al., 1994).

Regions B and C

The phenolic signal molecule located at HPLC region C (Fig. 2) was identified as dihydrowogonin 7-glucoside (Fig. 4). This was confirmed by the R_F value and brown color of the flavanone glucoside on TLC plates under UV light and by a UV spectrum with a prominent A_{max} at 286 nm and a minor broad A_{max} at 346 nm. Co-chromatography of the fraction with an authentic sample of dihydrowogonin 7-glucoside (Treutter et al., 1985) by HPLC resulted in an identical retention time. Hydrolysis of the compound yielded the aglucone, dihydrowogonin (A_{max} 290 and 334 nm). The phenolic signal(s) located at HPLC region B (Fig. 2) remains to be purified and identified.

syrB-Inducing Activities of Flavonoid Glycoside Signal Molecules from Cherry Leaves

The three flavonoid glycosides purified in large quantities from *P. avium* displayed similar *syrB*-inducing activity profiles at concentrations ranging from 0.1 to 100 μM (Fig. 5). High levels of signal activity were observed at 50 and 100 μM concentrations as noted by recoveries of more than

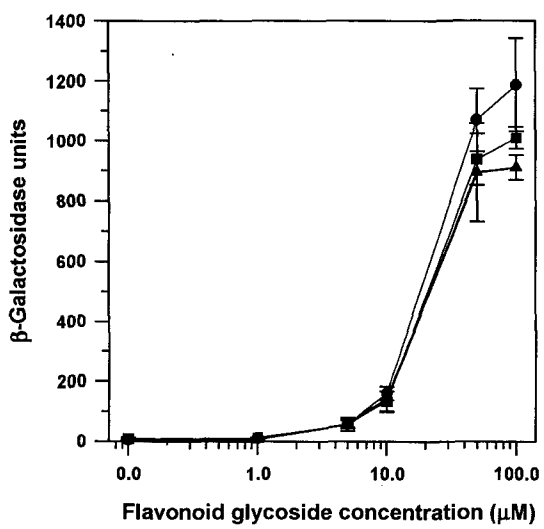

Figure 5. Effect of concentration of flavonoid glycosides isolated from *P. avium* on induction of the *syrB-lacZ* fusion in strain B3AR132 of *P. s. syringae*. β-Galactosidase activities were measured after incubation for 3 d in SRM liquid medium supplemented with 0.1 to 100 μM of dihydrowogonin 7-glucoside (●), kaempferol 3-rutinosyl-4′-glucoside (■), or quercetin 3-rutinosyl-4′-glucoside (▲). Each value is the mean of two trials conducted on separate occasions; each trial consisted of duplicate cultures. The background level of β-galactosidase activity was subtracted. Vertical bars indicate the SE.

900 units of β-galactosidase activity. Although only low activities of less than 15 units were observed at 1 μM, moderate activities averaging between 60 and 160 units were observed at 5 and 10 μM; such levels of signal activity were comparable to those reported for arbutin and a few other phenolic β-glucosides (Mo and Gross, 1991b). Dihydrowogonin 7-glucoside showed slightly higher activities than the two flavonol glycosides, but these differences were not significant. Insufficient quantities of quercetin 3,4′-diglucoside and kaempferol 3,4′-diglucoside were purified from cherry leaves for measurements of possible *syrB*-inducing activity.

The *syrB*-inducing activities of the three purified flavonoid glycosides from cherry were enhanced by about 10-fold by the addition (0.1%) of D-Fru (Table I). The two flavonol triglycosides, quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside, and the flavanone glycoside, dihydrowogonin 7-glucoside, had similar specific activities of *syrB* induction at 10 μM concentrations yielding more than 1300 units of activity when Fru was present; this represents between 380 and 800 units μg^{-1} of signal activity. Moreover, the *syrB*-inducing activities of the flavonoid glycosides were not significantly different from that of arbutin. In the absence of phenolic signals, D-Fru yielded about 100 units of intrinsic *syrB*-inducing activity.

The relationship of chemical structure to signal activity was examined by testing components and substituted derivatives of active flavonoid glycosides. Although the commercial availability of such compounds is limited, it appears that glucosylation at position 4′ in the flavonol signals is essential for activity based on a lack of signal activity for rutin (i.e. quercetin 3-rutinoside). Correspondingly, tests of quercetin 3-rhamnoside also failed to show *syrB*-inducing activity, indicating that glycosylation at position 3 of the flavonoid structure was insufficient for signal activity. The phenolic aglycone moieties, quercetin and kaempferol, of the flavonol signals were inactive as expected (Mo and Gross, 1991b).

DISCUSSION

The leaves of *P. avium* contain significant quantities of plant signal molecules that are perceived by *P. s. syringae* to activate the *syrB* gene necessary for syringomycin production and full virulence. All of the signal activity was soluble in water and was composed of two types of signal molecules, namely phenolic glycosides and sugars. The phenolic signals were separated by HPLC into three regions, which indicated that relatively few phenolic substances in cherry leaves display appreciable signal activity. Moreover, the phenolic signals in regions A and C were identified as flavonoid glycosides, a class of phenolics that had not yet been reported to exhibit *syrB*-inducing activity (Mo and Gross, 1991b). Two flavonol glycosides, quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside, occurred in region A, and the flavanone glucoside, dihydrowogonin 7-glucoside, occurred in region C. Dihy-

drowogonin 7-glucoside is the primary flavonoid occurring in the bark tissues of *P. avium* (Treutter et al., 1985). Relatively high amounts of dihydrowogonin 7-glucoside occur in tissues based on the recovery from phloem of more than 11 mg g^{-1} (dry weight) (Treutter et al., 1985, 1987).

In *P. avium* leaves, dihydrowogonin 7-glucoside is located exclusively in the main veins so that the concentration in the total leaf is significantly lower than in the bark (Gutmann, 1993). Typical for the phenolic composition of leaves is the occurrence of flavonols (Bauer et al., 1989). Because the other main flavonols (e.g. rutin and kaempferol 3-rutinoside) show no signal activity, the two flavonol triglycosides are the most important compounds with signal activity. We estimated that crude cherry leaf extracts contain more than 160,000 units g^{-1} (dry weight) of total signal activity, and this reflects both the relatively high quantities of flavonoid glycoside signals that occur in leaves and their corresponding specific signal activities estimated to be between 380 and 800 units μg^{-1} in the presence of D-Fru (Table I). It was not surprising to find that the total signal activity recovered from cherry leaves increased at each step in purification because crude plant extracts contain substances that are not active and also may inhibit expression of *syrB*, such as phenolic aglycones (Mo and Gross, 1991b; Quigley and Gross, 1994). Chlorogenic acid, the main phenolic compound in cherry leaves (Bauer et al., 1989), inhibits the growth of *P. s. syringae* in vitro by about 75% (Bayer, 1989). Nevertheless, induction of the *syrB-lacZ* fusion in strain B3AR132 by the crude extract was substantial, and this indicates that there are sufficient quantities of the flavonoid glycoside signals to induce phytotoxin production by *P. s. syringae* as it invades cherry leaves.

The signal activities of the flavonoid glycosides were markedly enhanced in the presence of sugars that commonly occur in high quantities in the leaves of *P. avium*. Suc and D-Fru were the most effective sugars, causing a 10-fold stimulation of signal activity when flavonoid glycosides occurred at low concentrations. In addition, Suc and D-Fru are the only sugars known to have intrinsic, but low level, *syrB*-inducing signal activity (Mo and Gross, 1991b). Consequently, the signal activity associated with the void volume in HPLC profiles is attributed to the presence of both sugars; this is substantiated by the occurrence of D-Fru in the sample containing flavonol glucosides extracted from paper chromatograms. Both Suc and Fru compose more than 3% of the dry weight of various tissues of *P. avium* (Schmid and Feucht, 1986; Keller and Loescher, 1989). These are ample quantities of sugars with signal activity for rapid and efficient induction of *syrB* in cherry tissues because Fru levels as low as 0.001% (w/v) enhance phenolic signal activity (Mo and Gross, 1991b). Although enhancement of flavonoid glycoside-mediated induction of *syrB* by specific sugars resembles the sensory mechanism responsible for activation of *vir* genes in *A. tumefaciens*, there are important differences in sugar specificities between the two systems because *vir* genes are not induced by Suc and D-Fru (Ankenbauer and Nester, 1990; Cangelosi et al., 1990; Shimoda et al., 1990; Mo and Gross, 1991b).

Our discovery that *P. avium* leaves contain flavonol and flavanone glycosides with signal activity demonstrates that *P. s. syringae* senses plant metabolites representative of distinct categories of phenolic compounds, including flavonoids, coumarins, and phenols (Mo and Gross, 1991b). A common structural feature of all *syrB*-inducing phenolic signal molecules is the presence of an intact glucosidic linkage. For example, all signal activity is lost if the glucosidic linkage of quercetin 3-rutinosyl-4'-glucoside is cleaved to yield rutin (i.e. quercetin 3-rutinoside); flavonoid aglycones, such as quercetin and kaempferol, likewise lack signal activity. Because plants generally accumulate and store phenolic compounds as glycosides to make them more water soluble and less chemically reactive (Hösel, 1981), phenolic glycosides would be more accessible and less likely to be inhibitory to *P. s. syringae*. Accordingly, Bayer (1989) observed that dihydrowogonin 7-glucoside, tested at 1 mM, was not inhibitory to the growth of *P. s. syringae*. In comparison to arbutin (Table I), the flavonol and flavanone glycoside signals from cherry leaves were found to be equally effective as signal molecules. Furthermore, the specific *syrB*-inducing activities of flavonoid glycosides from *P. avium* leaves, especially in the presence of soluble carbohydrates such as Fru, are comparable to the levels reported for plant metabolites with *vir*- or *nod*-inducing activity (Firmin et al., 1986; Melchers et al., 1989).

In earlier studies, it was established that a *syrB-lacZ* gene fusion can be used to measure environmental factors that influence expression of syringomycin synthesis (Mo and Gross, 1991a, 1991b). Accordingly, it proved in this study to be a convenient and powerful tool for assaying signal activity extracted from leaves of *P. avium*. The relationship of plant signal molecules to toxigenesis in *P. s. syringae* was substantiated by the conservation of the *syrB* gene in all toxigenic strains and by induction of higher levels of syringomycin production in almost all toxigenic strains (Quigley and Gross, 1994). It now appears that the plant-inducible *syrB* gene encodes an adenylate-forming enzyme that activates and binds one of the structural amino acids of syringomycin and that *SyrB* participates in a thiotemplate mechanism of peptide synthesis (J.-H. Zhang, N.B. Quigley, and D.C. Gross, unpublished data). Because other *syr* loci, including *syrC*, appear to be transcriptionally activated by chemical stimuli of plant origin (Mo and Gross, 1991a, 1991b), it would not be surprising to discover that other virulence genes of *P. s. syringae* are controlled by the same signal transduction process. Consequently, the pathogenicity of *P. s. syringae* in cherry leaves would be modulated by the perception of flavonoid glycosides and sugars that serve as signal molecules.

The control of toxigenesis in *P. s. syringae* by plant signal molecules reflects its ability to adapt to a dynamic plant environment. A common predisposing factor to infection of cherry by *P. s. syringae* is frost injury (Zeller and Schmidle, 1979; Gross et al., 1984). The occurrence of an injurious frost would lead not only to wounding of cherry tissues and leakage of nutrients but also to the sudden release of phenolic glycoside signals, the bulk of which are compart-

mentalized in vacuoles (Hösel, 1981). Because high amounts of flavonoid glycoside signals occur in *P. avium* leaves, one can speculate that their sudden release would be quickly sensed by the bacterium and lead to activation of genes, such as *syrB*, necessary for phytotoxin production. In addition, it appears that a broad spectrum of *P. s. syringae* strains would be capable of recognizing the flavonoid glycoside signals from *P. avium*. This is based on evidence that most strains of *P. s. syringae* attack a wide range of plant hosts (Bradbury, 1986) and that they recognize phenolic signal molecules that are found in the leaves, bark, and flowers of many plant species (Mo and Gross, 1991b; Quigley and Gross, 1994). Moreover, the flavonol glycoside signals, quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside, were reported by Saleh et al. (1983) to occur in five species of *Erodium* (Geraniaceae subsection *Malacoidea*) as the major flavonoid glycosides in foliar tissues. Therefore, the occurrence of a sensory mechanism for specific phenolic glycosides is advantageous because it signals the bacterium to activate rapidly virulence genes in a favorable plant environment. Correspondingly, host plants harbor phenolics with the fundamental chemical structures for signal activity to sustain an aggressive attack by the pathogen. Host resistance as observed, for example, in cherry cultivars (Krzesinska et al., 1993) may reflect qualitative and quantitative differences in signal molecules or the balance of plant substances that antagonize induction by plant signals.

ACKNOWLEDGMENT

We thank Dr. Linda Thomashow for use of HPLC equipment.

Received September 9, 1994; accepted November 7, 1994.

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