Inducing the *syrB* Gene in *Pseudomonas syringae* pv. *syringae* in Twig Extracts from Cherry Genotypes

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Abstract. The *syrB* gene required by *Pseudomonas syringae* pv. *syringae* van Hall to produce the phytotoxin syringomycin is activated by plant signal molecules. Extracts from twigs of 12 cherry (*Prunus*) genotypes were tested for their ability to induce *syrB::lacZ* fusion in *P. syringae* pv. *syringae* strain B3AR132 to determine whether signal activity is correlated with susceptibility to bacterial canker. One-year-old twigs of 'Napoleon', 'Corum', and 12 cherry rootstocks (F12/1, 'Colt', MxM2, MxM39, MxM60, Gi 148-1, Gi 148-9, Gi 154-2, Gi 154-5, Gi 169-15, Gi 172-9, and Gi 173-9) were tested at concentrations of 0.2, 1.0, and 2.0 mg twig dry weight/ml solution for their ability to induce *syrB::lacZ* fusion, as measured by β -galactosidase activity. Extracts from all cherry genotypes induced *syrB::lacZ* fusion, but to varying degrees. The highest β -galactosidase activity was observed in 'Napoleon' and 'Corum'—the most susceptible genotypes; activities were two to four times higher than that of F12/1, a disease-resistant genotype. Activities higher than that of F12/1 were induced at the lowest extract concentration by rootstocks MxM60, Gi 148-1, Gi 148-9, and Gi 154-5, whereas rootstocks MxM2, MxM39, Gi 154-2, Gi 172-9, Gi 173-9, and 'Colt' were not significantly different from F12/1. At the two highest extract concentrations tested, only 'Napoleon' and 'Corum' consistently had higher induct&m activity than F12/1. At high extract concentrations, interfering substances seemed to suppress or antagonize the induction of *syrB::lacZ* fusion. These results suggest that susceptible genotypes contain higher signal activities than resistant genotypes.

In the Pacific Northwest, bacterial canker caused by *Pseudomonas syringae* pv. *syringae* is a serious disease of sweet cherries (*Prunus avium* L.). Above-ground portions of trees are infected during the region's cool, wet season from fall until early spring. Infection may produce localized cankers and kill buds, limbs, or the entire tree (Cameron, 1962a, 1962b). Leaf and fruit lesions are observed rarely.

Pseudomonas syringae pv. *syringae* produces syringomycin, an onhost-specific cyclic lipodepsinonapeptide phytotoxin (Gross, 1991; Segre et al., 1989). This toxin disrupts ion transport across the plasmalemma of affected cells and leads to necrosis (Bidwai et al., 1987;

Mott and Takemoto, 1988). Syringomycin production contributes significantly to virulence by increasing disease severity (Xu and Gross, 1988a, 1988b).

Five proteins are associated with syringomycin production and are believed to function as syringomycin synthetases (Gross, 1991; Morgan and Chatterjee, 1988). The formation of two large proteins, SR4 and SR5, is associated with the *syrB* gene (Xu and Gross, 1988b). *SyrB* either encodes a subunit of multimeric synthetase protein or is a positive regulatory protein that controls synthetase expression (Mo and Gross, 1991a). To study the expression of syringomycin production, Mo and Gross (1991a) mutagenized the *syrB* gene with Tn3HoHo1 to generate fusions to a promoterless *lac* operon. A transcriptional fusion of *syrB* with *lacZ* genes resulted in high β -galactosidase activity and was used to quantify *syrB* expression. The *syrB::lacZ* fusion was recombined into the genome of *P. syringae* pv. *syringae* strain B3A-R, which does not produce syringomycin in syringomycin minimal (SRM) medium (Mo and Gross, 1991a). The resulting recombinant B3AR132 also lacked β -galactosidase activity in SRM medium. However, adding cherry leaf extracts to SRM medium induced expression of *syrB::lacZ* fusion in strain B3AR132 (Mo and Gross, 1991a).

This virulence gene, which is partly responsible for syringomycin production by *P. syringae* pv. *syringae*, may be induced by compounds produced in the host plant's leaves.

Cherry trees grafted high (100 cm) on a tolerant rootstock, such as F12/1, sometimes have fewer cankers in the scion than lower grafts (Cameron, 1971). High grafting currently is recommended to reduce disease incidence. Several interspecific hybrid rootstocks, the MxM and Gisela clones, are being evaluated for horticultural acceptability in the United States. In an earlier study, the susceptibility of many of these rootstocks to *P. syringae* pv. *syringae* was determined in a twig assay (Krzesinska and Miller-Azarenko, 1992). In the study we report here, our objective was to test whether extracts of 1-year-old wood of various cherry genotypes with different susceptibilities to *P. syringae* pv. *syringae* would induce the expression of *syrB::lacZ* gene fusion in strain B3AR132.

Ten 'Napoleon' sweet cherry twigs were inoculated in Mar. 1990 with 10⁶ colony forming units (cfu)/ml of strain B3AR132 to determine if this strain could induce the same symptoms as the four virulent strains used in an earlier twig assay (Krzesinska and Miller-Azarenko, 1992). A 20- μ l droplet of inoculum was placed onto a 0.5 $\times$ 2-cm flap cut into the twig to expose the cambium. Twigs (1-year-old wood, 15 cm long) were placed into sterile test tubes on water-saturated cotton, covered, and incubated for 4 weeks at 15C in darkness. Incision browning (1 = yellow pith, 4 = dark brown) was assessed after incubation and used to score strain virulence. After 4 weeks of incubation, browning and gummosis were observed on 'Napoleon' twigs inoculated with strain B3AR132; thus, B3AR132 was pathogenic and retained at least some virulence. Therefore, it was used to evaluate cherry rootstock genotypes with different levels of disease resistance for relative plant signal activities.

One-year-old wood was collected from trees of 'Napoleon', 'Corum', F12/1, 'Colt', MxM2, MxM39, MxM60, Gi 148-1, Gi 148-9, Gi 154-2, Gi 154-5, Gi 169-15, Gi 172-9, and Gi 173-9 genotypes in Mar. 1990. 'Napoleon', 'Corum', and F12/1 are *P. avium* selections. 'Napoleon' and 'Corum' were susceptible controls and F12/1 was a resistant control. The MxM rootstocks are interspecific *P. avium* $\times$ *P. mahaleb* L. hybrids. The Gi series was selected from interspecific crosses between *P. avium*, *P. cerasus* L., *P. canescens* Bois., and *P. fruticosa* Pall. (Cummins, 1984). Twigs were washed in water, air-dried, cut in 1.0-cm pieces, frozen in liquid nitrogen, and lyophilized. The dried samples then were ground in a Wiley mill, passed through a 40-mesh screen, and stored at -20C.

Water extracts were prepared from lyophilized ground twig samples of the various *Prunus* genotypes. Tissue weights of 20, 100, and 200 mg were homogenized in a Waring blender with 100 ml of double-distilled water. For each genotype and concentration, 2 ml of aqueous extract was evaporated to dryness and

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resuspended in 2 ml of 75% acetone to remove proteins. After 30 min, the samples in 75% acetone were evaporated to dryness, resuspended in 2 ml of distilled water, filter-sterilized, and stored at -20°C. Each genotype was assayed three times, with two replications each time.

Twig aqueous extracts were assayed for their effects on the expression of β -galactosidase activity by *sydB::lacZ* fusion in B3AR132 as described by Mo and Gross (1991a). Plant extracts (100 μ l) containing 1, 5, or 10 mg (dry weight) of sample were added to 4.9 ml of SRM medium containing 10^6 cfu/ml of *P. syringae* pv. *syringae* mutant strain B3A132. Duplicate SRM cultures also were prepared with the parental strain B3A-R or without bacteria; these cultures were the controls and were used to measure background activity in the assay. After being shaken and incubated for 3 days at 25°C, cells from 0.5 ml of culture were pelleted in microcentrifuge tubes, suspended in Z-buffer (1.4 ml), repelleted, and resuspended in 1.4 ml Z-buffer (Miller, 1972). A portion of the washed cells (1 ml) was measured for bacterial cell density at 600 nm. Twenty microliters of 0.05% sodium dodecyl sulfate and 20 μ l of chloroform were added to 0.5 ml of the washed cells. The cells were lysed by vortexing them for 10 sec and then incubating them for 10 min at 28°C. The assay reaction was started by adding 100 μ l of *o*-nitrophenyl- β -D-galactopyranoside (4 mg·ml⁻¹) to the culture and then incubating it for 10 min at 28°C. The reaction was terminated by adding 250 μ l of 1M Na₂CO₃. Cellular debris was removed by pelleting the culture in a microcentrifuge; the absorbency of the solution was determined at 420 nm. Miller units of β -galactosidase activity were calculated using the formula given by Stachel et al. (1985).

Plant substances present in cherry twigs induced expression of *sydB::lacZ* fusion in B3AR132 (Fig. 1). Activity varied with rootstock. In general, as the concentration of cherry extracts increased, the β -galactosidase activity decreased. This result was attributed to the presence of substances in crude extracts, which, at higher concentrations, inhibited *sydB* induction or antagonized the effect of the plant signals. Twig extracts containing no bacteria in the SRM medium had background β -galactosidase activity similar to that of the extracts with the nonsyngomycin-producing parental strain B3A-R (data not shown).

Compared to F12/1, 'Napoleon' and 'Corum' had the highest activity, regardless of plant extract concentration (Fig. 1). When inoculated with the lowest concentration of plant extract, rootstocks MxM2, MxM39, Gi 154-2, Gi 172-9, Gi 173-9, and 'Colt' were not different from F12/1. The β -galactosidase activity was higher for extracts from rootstocks MxM60, Gi 148-1, Gi 148-9, Gi 154-5, and Gi 169-15 than those from F12/1.

At the two higher concentrations, extracts from rootstocks MxM39, Gi 148-9, Gi 154-2, Gi 154-5, Gi 169-15, Gi 173-9, and 'Colt' had lower activity than F12/1. Compared to F12/1, rootstocks MxM2, Gi 148-2, and Gi 172-9 were not different at an extract concentration

Fig. 1. The effect of rootstock genotype and extract concentration on recovery of signal activity as measured by expression of β -galactosidase activity from the *sydB::lacZ* fusion of *Pseudomonas syringae* pv. *syringae* strain B3AR132. Activities are compared to those of the resistant rootstock genotype F12/1. Each genotype was assayed on three occasions with two replications each time. NS, *, **, *** = Nonsignificant or significant at $P = 0.05$, 0.01 , or 0.001 , respectively.

of 1.0 mg·ml⁻¹, but activity was higher at 2.0 mg·ml⁻¹. The lowest activity was induced by the rootstock 'Colt'.

Since variable results were obtained at different concentrations, further purifying the crude extracts may improve the assay's accuracy by removing interfering substances. Additionally, inhibitors may be identified using the method described for plant signal induction in *Agrobacterium tumefaciens* (E. F. Sm. & Town.) Conn. (Djordjevic et al., 1987; Sahi et al., 1990) and *Rhizobium meliloti* Dang. (Peters and Long, 1988).

In many cases, degree of incision browning in an excised twig assay, as observed in an earlier study with some of the same rootstocks (Krziesinska and Miller, 1992), paralleled the degree of *sydB* induction. In both assays, 'Napoleon' and 'Corum' had the highest browning and β -galactosidase activity. Rootstock Gi 169-15 at the lowest extract concentration

induced high β -galactosidase activity and also was more susceptible in the twig assay. Differences were observed with rootstocks MxM60, Gi 148-1, Gi 148-9, and Gi 154-5, which were similar to F12/1 in the earlier twig assay but had high activity at low extract concentrations in the present bioassay. The opposite situation was observed for rootstock Gi 172-9, which seemed to be susceptible to *P. syringae* pv. *syringae* in the twig assay (Krziesinska and Miller-Azarenko, 1992) but not different from F12/1 in β -galactosidase activity. In both assays, the other rootstocks did not differ from F12/1 in susceptibility. One explanation for these differences in the assays may be the physiological stage of the twigs. For the bioassay, twigs were collected only once, in Mar. 1990, whereas samples were collected earlier in the winter for disease susceptibility tests.

The cherry substances serving as *sydB*-inducing plant signals remain to be identified.

Nevertheless, Mo and Gross (1991b) identified some phenolic glycosides that function as signal molecules in *syrB* induction in *P. syringae* pv. *syringae*. The structural requirement for maximum *syrB* induction is a glucosylated benzene ring. Phenolic compounds generally are stored as glycosides to increase water solubility and decrease reactivity (Hösel, 1981). Many phenolic glucosides are found in plant species within the host range of *P. syringae* pv. *syringae* (Miller, 1973; Paris, 1963). Additionally, sugars such as sucrose and fructose enhance the response of *P. syringae* pv. *syringae* to the phenolic signal (Mo and Gross, 1991a). Sugars seem to be an integral part of the signal transduction process.

The results may vary with sampling time, since there may be seasonal fluctuation in the concentration of plant signals or substances that antagonize induction by plant signals. Identifying the signal molecules that induce phytotoxin production by *P. syringae* pv. *syringae* and determining seasonal fluctuation in their concentrations may lead to a better understanding of the host-pathogen interaction and provide a basis for more effective disease-control practices.

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