Identification of an *Agrobacterium tumefaciens* virulence gene inducer from the pinaceous gymnosperm *Pseudotsuga menziesii*

(crown gall tumors/coniferin/coniferyl alcohol/glucosidase)

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ABSTRACT Inducible T-strand mobilization from the Ti plasmid of *Agrobacterium tumefaciens* to the genome of a plant host is mediated by the activation of a cascade of bacterial virulence genes. It is initiated when the bacterium senses the presence of a low molecular weight inducer secreted by the plant. Although many hydroxyphenylpropanoid and phenolic compounds can activate the virulence cascade, the only native inducers that have been identified to date are acetosyringone and hydroxyacetosyringone. A new inducer, the phenylpropanoid glucoside coniferin, has now been isolated from *Pseudotsuga menziesii* (Douglas-fir). *Agrobacterium* strains that were more tumorigenic on gymnosperms were more effectively induced by coniferin.

Agrobacterium tumefaciens, the causal agent of crown gall disease, is now widely known for its ability to transfer part of its DNA (the T-DNA) from the bacterial tumor-inducing (Ti) plasmid to the genome of the plant host (for recent reviews see refs. 1 and 2). Substantial interest has focused on this bacterium because it provides one of the more efficient methods for introduction of chimeric genes of economic importance into crop plants. However, not all plants can be successfully transformed (3). Different *A. tumefaciens* strains exhibit strong host specificity even among dicotyledonous plants (4) and many plants are refractory. Thus, monocotyledonous species are recalcitrant to transformation and when they can be transformed, it is only at low frequency (5, 6). Gymnosperms have also been considered to be resistant to infection by *A. tumefaciens*, although recent work (7–9) has shown that they are susceptible to infection at low frequency.

Successful transformation requires the induction of a cascade of bacterial virulence genes present on the Ti plasmid. The process is believed to begin when a plant phenolic inducer (10) interacts with the *A. tumefaciens* *virA* gene product. Subsequently the *virG* gene product is activated and it, in turn, activates the expression of the other virulence genes. The process culminates in packaging the T-DNA, transfer to the plant, and integration into the plant nuclear genome. Several plant phenolic compounds have been shown to initiate the first steps of this cascade (11–13). Nevertheless, the only native inducers that have been identified are acetosyringone and hydroxyacetosyringone, which were isolated from wounded tobacco leaves and tobacco root cultures (14).

Recent experiments (15) indicated that certain *A. tumefaciens* strains could transform conifers at high frequency. Other strains, which were equally tumorigenic on herbaceous species, were much less tumorigenic on conifers. However, it was not known whether this specificity arose because gymnosperms possessed a set of phenolic inducers that differed chemically from those of herbaceous species or was

due to other causes. Virulence-inducing compounds can readily be assayed by using the reporter plasmid pSM358 (12, 16, 17) in *A. tumefaciens*. The plasmid contains a translational fusion of the *Agrobacterium* *virE* gene and the *Escherichia coli* *lacZ* gene that, in the presence of virulence-region inducers and the other genes of the virulence cascade, expresses β -galactosidase activity. Using this plasmid we were able to isolate and characterize the major native phenolic virulence-gene inducer from *Pseudotsuga menziesii* (Douglas-fir).

MATERIALS AND METHODS

Plant Material and Bacterial Strains. Lateral shoots (terminal 10 cm) of the current season's growth were collected in May from young (20 yr), actively growing *P. menziesii* trees located in Corvallis, Oregon, and were stored at 4°C for 4 days prior to extraction of phenolic compounds.

A number of wild-type *Agrobacterium* isolates were obtained from different sources: MFM83.4 from M.-F. Michel (Station d'Amelioration des Arbres Forestiers, Institut National de la Recherche Agronomique, Orleans, France); strains 3667, B3/73, K41, K47, T28/73, and RR5 from L. Moore (U.S. Department of Agriculture Ornamentals Laboratory, Corvallis, OR); A518 and C58 from E. Nester (Microbiology Department, University of Washington, Seattle). The reporter fusion plasmid, pSM358, was provided by P. Zambryski (Molecular Plant Biology Department, University of California, Berkeley). It was introduced into *Agrobacterium* strains by the triparental mating procedure of Ditta *et al.* (18).

Tissue Extraction and Initial Fractionation. A methanolic extract of crushed *P. menziesii* shoots was prepared by incubation of the tissue (300 g) in 80% (vol/vol) methanol (2 liters) for 18 hr followed by rotary evaporation to 1% of the original volume. An aliquot of the extract was fractionated by reversed-phase HPLC (250 $\times$ 4.6 mm column, 5- μ m Ultrasphere ODS, Altex) with gradient elution by 10–90% acetonitrile in 40 mM triethylammonium acetate (pH 3.35) over 40 min. Fractions were assayed for virulence-inducing compounds by addition to *Agrobacterium* cultures and subsequent measurement of β -galactosidase activity.

Virulence Gene Induction Assay. The assay for β -galactosidase was adapted from Miller (19). Logarithmic-phase liquid cultures were diluted (OD₆₅₀ 0.035) into 100 μ l of a medium (VIM4) containing, per liter, KH₂PO₄, 1.0 g; Murashige and Skoog salt mixture (GIBCO), 0.5 g; MgSO₄·7H₂O, 0.1 g; NaCl, 0.2 g; (NH₄)₂SO₄, 1.0 g; yeast extract (Difco), 1.0 g; CaCl₂·2H₂O, 0.07 g; glycerol, 10 ml; pH was adjusted to 5.6. Aliquots of HPLC fractions (5–50 μ l evaporated to dryness and redissolved in 1 μ l of dimethyl sulfoxide) or acetosyringone (200 mM, 1 μ l) were added. After 12 hr, cells were centrifuged, the cell pellet was

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Abbreviation: TMS, trimethylsilyl.

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resuspended in 100 μ l of VIM4, and the culture density was determined. Enzyme activity was assayed after the addition of 0.2 volume of a 5-fold concentrate of Z buffer (19), modified by deletion of the chloroform and increase of sodium dodecyl sulfate to 0.1% (wt/vol). Following incubation (10 min, 22°C), *o*-nitrophenyl β -D-galactopyranoside (5.8 mM; Sigma) was added, and color development was monitored for 2 min at 405 nm (VMAX Microplate Reader, Molecular Devices, Palo Alto, CA). β -Galactosidase activities were expressed as nmol/min per unit of cell culture density (OD₆₅₀). When the specific inducibility of coniferin and the isolated inducer were compared, equal concentrations (5–500 μ M, based on UV absorbance) were used. When the induction of different strains was compared (see Table 1), 200 μ M coniferin was used.

Purification of the Active Inducer. Crude concentrated methanolic extract was diluted with an equal volume of 10 mM ascorbic acid (pH 6.5) and partitioned sequentially against hexane, ethyl ether, and ethyl acetate. The aqueous phase was passed through an octadecyl-silica column (Separylite 40 μ m; Analytichem International, Harbor City, CA; 80 $\times$ 10 mm), which was then washed with 10 mM ascorbic acid (pH 6.5). Active compounds were eluted with two column volumes of methanol. The eluate was fractionated by preparative chromatography on octadecyl-silica (40 μ m, 100 $\times$ 20 mm) followed by reversed-phase HPLC (conditions as above except that elution was with methanol). Active fractions were combined and rechromatographed under isocratic conditions in 20% methanol for final purification.

β -Glucosidase Hydrolysis of Inducers. After purification, two aliquots of the active material were evaporated to dryness, and one was redissolved in 100 μ l of 20 mM sodium acetate (pH 5.0) containing 1.0 unit of β -glucosidase (Sigma). After incubation (1 hr, 37°C), the enzyme was precipitated with 3 volumes of methanol, the sample was centrifuged, and the supernatant was evaporated to dryness. The dried samples were rechromatographed as above, and HPLC fractions were assayed for induction activity.

Structure Determination. Trimethylsilyl (TMS) derivatives were prepared using TMSlimidazole (Pierce) in anhydrous pyridine. Electron-impact spectra were acquired on a Kratos MS 50 S mass spectrometer (Kratos Analytical Instruments) interfaced with a Carlo Erba model 4160 gas chromatograph. Spectra were recorded at 70 eV with an ionization current of 50 μ A, a source temperature of 250°C, and a transfer temperature of 290°C. The gas chromatograph was fitted with an OV-7 fused silica capillary column (60 m $\times$ 0.25 mm) operating under isothermal conditions at 290°C with injector and detector temperatures at 310°C.

Nuclear magnetic resonance spectra were acquired using a Nicolet NT 300-WB spectrometer operating at 300.06 MHz and equipped with a 5-mm proton probe. Transients were accumulated using a 9- μ sec pulse and 1-sec relaxation time. Field shifts were referenced to tetramethylsilane and the solvent ([²H₄]methanol).

β -Glucosidase Assay in *A. tumefaciens*. The glucosidase assay was adapted from the virulence gene induction assay (above). Bacterial cultures (initial density, OD₆₅₀ 0.020) were grown 18 hr in VIM4 containing 200 μ M coniferin and then mixed with 0.2 volume of 5 $\times$ modified Z buffer. After incubation (10 min, 22°C), *p*-nitrophenyl β -D-glucopyranoside (Sigma) was added (5.8 mM). Color development was monitored for 15 min at 405 nm. β -Glucosidase activity was expressed as nmol/min per unit of cell culture density.

RESULTS

The marker plasmid pSM358 (16) provided a useful and convenient assay for the presence of virulence gene inducers. Enhanced expression of β -galactosidase from the *virE-lacZ*

fusion of pSM358 indicated the presence of an active inducer. The plasmid was introduced into two *Agrobacterium* strains. The first, B3/73, was strongly tumorigenic on *P. menziesii* (15). The second, MFM83.4, was only weakly tumorigenic. When these strains were used to assay HPLC fractions of phenolic compounds extracted from *P. menziesii*, a differential response was seen (Fig. 1). Both strains gave approximately equivalent responses to acetosyringone, but the strongly tumorigenic strain B3/73(pSM358) responded to a compound (PM1) present in the crude *P. menziesii* extract. The weakly tumorigenic strain MFM83.4(pSM358) did not respond.

The retention time of PM1 did not correspond to that of any previously characterized *Agrobacterium* virulence inducer. In order to identify PM1, a large-scale preparation of Douglas-fir phenolic compounds was undertaken. Fresh shoots were extracted with methanol and the extracts were subjected to solvent partitioning and HPLC. Active compounds were not soluble in hexane or ether but could be purified by adsorption to octadecyl-silica followed by elution with methanol. Assay of HPLC fractions with B3/73(pSM358) established that at least three active compounds were present. The first, compound PM1, possessed the bulk of the biological activity and was selected for further purification.

HPLC fractionation of PM1 on octadecyl silica columns provided homogeneous material possessing biological activity. A typical HPLC profile of purified PM1 is illustrated in Fig. 2 A and C. The compound had a retention time of 17.2 min, considerably shorter than that of acetosyringone (31.0 min) or of any other known phenolic inducer, suggesting that it was quite polar. In light of this polarity it seemed possible that it might be a glucoside. Treatment with β -glucosidase confirmed this supposition (Fig. 2 B and D). After glucosidase treatment, full activity was retained but the retention time of the active species increased to 28.0 min. The retention time of the hydrolysis product coincided with that of authentic coniferin alcohol.

The structure of PM1 was determined from mass spectra and proton nuclear magnetic resonance spectra. The TMS derivative gave a homogeneous peak on gas chromatography with a retention time identical to that of authentic TMS-coniferin. The electron-impact mass spectra of TMS-PM1

FIG. 1. Assay of HPLC fractions for *P. menziesii* virulence-inducing compounds with strongly and weakly tumorigenic *Agrobacterium* strains. β -Galactosidase activity is expressed as nmol of *o*-nitrophenyl β -D-galactopyranoside hydrolyzed per minute per unit of cell density. (A) Assay with the strongly tumorigenic strain B3/73(pSM358). Active material was eluted at 12.5 min. (B) Assay with the weakly tumorigenic strain MFM83.4(pSM358). No significant induction was detected. Activity induced by 200 μ M acetosyringone is indicated by the bars at 41 min.

FIG. 2. HPLC and activity of purified compound PM1 before and after treatment with β -glucosidase. (A and C) Untreated PM1: absorbance at 254 nm and virulence induction activity with strain B3/73(pSM358). (B and D) PM1 after β -glucosidase treatment: absorbance at 254 nm and virulence induction activity. HPLC conditions were identical to those for analytical isolation except the methanol gradient was from 15% to 50% (vol/vol). Virulence induction was assayed as in Fig. 1. AS, acetosyringone; CA, coniferyl alcohol; SNA, sinapic acid; SYA, syringic acid.

and TMS-coniferin were identical (Fig. 3). TMS-PM1 had a base peak at m/z 73 and major fragment ions at m/z 103, 147, 217, 324, and 361. The spectrum of TMS-coniferin also contained the same peaks with the same relative intensities. The proton magnetic resonance spectrum of PM1 confirmed the structural assignment. Resonances were present at chemical shifts (ppm) -6.9 to -7.15 (ring protons), -6.3 to -6.6 (exocyclic protons), and -3.9 (ring methoxy protons). The spectrum was identical to that of coniferin.

To demonstrate that the biological activity of PM1 was intrinsic to the coniferin present and had not merely been copurified with it, the concentration dependencies of virulence gene induction in strain B3/73(pSM358) by PM1 and by coniferin were compared (Fig. 4). Activity profiles were not different within experimental error. On the basis of HPLC and gas chromatographic retention times, mass and proton magnetic resonance spectra, and biological activity, the major *A. tumefaciens* virulence-inducing substance found in young *P. menziesii* shoots is therefore coniferin.

As a glucoside, coniferin has a unique position among *Agrobacterium* virulence gene inducers. All previously characterized phenolic inducers are aglycones and possess free phenolic hydroxyl groups. Therefore, the question arose as to whether coniferin was the proximal inducer or whether it was first converted by the bacteria to coniferyl alcohol, which has

been shown to induce the *Agrobacterium* virulence region (12).

To investigate this question and the potential role of *Agrobacterium* glucosidases in the virulence induction process, a set of wild-type *Agrobacterium* strains that exhibited a range of tumorigenicities on conifers were transformed with the marker plasmid pSM358 and examined for virulence gene induction and glucosidase activity in the presence of coniferin. The data (Table 1) indicated that glucosidase activity was high in strongly tumorigenic *A. tumefaciens* strains and low in weakly tumorigenic strains. The least infective *Agrobacterium* strains exhibited no detectable glucosidase activity. Virulence gene induction correlated with glucosidase activity. Strains with high glucosidase activity exhibited high virulence gene induction that increased with time. The exception was strain 3667, which showed no induction.

Differential virulence gene induction by coniferin in strains B3/73(pSM358) and MFM83.4(pSM358) was not due to differential sensitivity to coniferyl alcohol. Acetosyringone, coniferin, and coniferyl alcohol all induced *vir* function in B3/73(pSM358), whereas in MFM83.4(pSM358), only acetosyringone and coniferyl alcohol were effective (Fig. 5). The ability of coniferin to act as a *vir* inducer correlates with the presence of glucosidase activity. HPLC analysis of culture supernatants showed that coniferin was being converted to coniferyl alcohol (data not shown).

FIG. 3. Mass spectra of TMS derivatives of PM1 and authentic coniferin. (A) Coniferin. (B) Compound PM1. (Inset) Structure of coniferin.

FIG. 4. Relative induction of B3/73(pSM358) virulence activity by PM1 (●) and coniferin (◇) over a range of concentrations. Virulence induction was assayed as in Fig. 1. Coefficient of variation of triplicate samples was 0.46.

DISCUSSION

Coniferin, a phenylpropanoid glucoside, is clearly an efficient inducer of the virulence regions of gymnosperm-specific *A. tumefaciens* strains. In addition to coniferin, there are at least two other inducers in extracts of *P. menziesii* (data not shown). These inducers, which have not been identified, are present at lower concentrations than coniferin but are also glucosides. That a glucoside is an efficient inducer of *A. tumefaciens* virulence-region genes raises interesting questions as to the nature of all native inducers and the potential role of processing enzymes, such as β -glucosidase, in the induction process. An attractive hypothesis is that the proximal inducer of the virulence region in these strains is coniferyl alcohol. Those strains that can respond to coniferin do so because they possess a glucosidase capable of converting coniferin to coniferyl alcohol. If this proves correct, it would be an interesting example of conversion by a pathogen of a normal plant metabolite to a signal capable of initiating a pathogenic process. With the exception of RR5, strains that exhibited no detectable β -glucosidase activity eventually responded slightly to coniferin as an inducer (Table 1, 18-hr time point), suggesting that a low-level β -glucosidase activity might be present. Preliminary cloning experiments (L. A. Castle and R.O.M., unpublished data) indicate that there may be two glucosidase genes present, one considerably more active than the other. The exact role

Table 1. Glucosidase activity and virulence gene induction in the presence of 200 μ M coniferin

Strain*	Tumorigenicity†	Glucosidase	vir induction		
			9 hr	12 hr	18 hr
K47	75	4.9	185	450	708
B3/73	72	3.0	160	150	490
3667	65	2.1	38	33	26
K41	60	1.9	45	67	195
A518	36	<0.3	5	7	25
K26	31	<0.3	2	10	82
T28/73	25	<0.3	3	34	33
C58	14	<0.3	7	34	46
MFM83.4	8.8	<0.3	6	23	69
RR5	6.3	<0.3	9	9	9

Glucosidase activity was measured at 18 hr, and vir induction (β -galactosidase activity) at 9, 12, and 18 hr. Enzyme activities are expressed as nmol/min per unit of cell density (OD_{650}). Coefficient of variation for triplicate samples was 0.10 for glucosidase measurements and 0.07 for vir gene induction.

*All strains harbored plasmid pSM358, and all strains were *A. tumefaciens* except K47 (*Agrobacterium rhizogenes*).

†Percentage of seedlings forming galls. Data are from ref. 15.

FIG. 5. Induction of virulence activity in *A. tumefaciens* B3/73(pSM358) (A) and MFM83.4(pSM358) (B) by coniferin (Δ), coniferyl alcohol ($\circ$), and acetosyringone ($\diamond$). Virulence assay was as in Fig. 1. Coefficient of variation of quadruplicate samples was 0.10.

played by these glucosidases and the validity of the conversion hypothesis remain to be determined.

Until very recently, all known *A. tumefaciens* inducers were aglycones. For example, the native inducers from tobacco are acetosyringone and hydroxyacetosyringone (14), although many other structurally related compounds are capable of induction and are effective at or below micromolar concentrations (12–14). The common structural features of these aglycone inducers are a free phenolic hydroxyl group, at least one methoxy group ortho to the hydroxyl, and an exocyclic electron-withdrawing group. The exceptions are coniferyl and sinapyl alcohols, which have ring hydroxyl and methoxy groups but which possess electron-donating propenyl side chains. In the experiments reported here, acetosyringone and coniferyl alcohol were found to be equally effective inducers of all strains, whereas coniferin was an effective inducer only of those *Agrobacterium* strains that possessed an active glucosidase. In a recent paper, Zerback *et al.* (17) demonstrated that several flavonol glycosides and one flavone glucoside were inducers of the *A. tumefaciens* virulence genes at millimolar concentrations. These inducers were 200 times less effective than acetosyringone on a molar basis. The test strain, A348(pSM358), used by Zerback *et al.* possesses low glucosidase activity (unpublished data). It would be of interest to determine the effectiveness of the flavonol and flavone glycosides on *A. tumefaciens* strains that possess high glucosidase activity.

There are interesting parallels between *A. tumefaciens* virulence gene induction and *Rhizobium* nodulation gene induction. Generally, *Rhizobium* nodulation inducers are flavones and isoflavones, $C_6-C_3-C_6$ diphenylpropanoids that contain a central oxygen-heterocyclic ring with an exocyclic keto oxygen. Like effective virulence inducers, effective nodulation inducers such as the flavone luteolin (20) and the isoflavone daidzein (21) contain an aromatic ring hydroxylated para to an electron-withdrawing group, the keto oxygen of the central ring. The second aromatic ring confers additional activity or specificity when hydroxylated. One flavone glucoside, apigenin 7-O-glucoside, has been reported to induce *Rhizobium leguminosarum* nodulation genes (22).

In light of the similarities between the two systems, the identification of glucoside inducers for both *A. tumefaciens* and *Rhizobium* species raises the question as to the role of glucosidases in the induction processes. The data from this study indicate an association of glucosidase expression, induction, and tumorigenicity on conifers. A causative role has not been demonstrated. Suitable mutagenesis and complementation studies must be performed to rigorously establish this point. Whether flavone glucosides can generally act as virulence or nodulation inducers and whether glucosidase activity is causally related to *Agrobacterium* tumorigenicity and activation of the *Rhizobium* symbiotic process remain to be determined.

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