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Salicylate Activity. 3. Structure Relationship to Systemic Acquired Resistance

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Salicylic acid (2-hydroxybenzoic acid; SA) is a primary signal inducing plant defenses against pathogens. This plant disease resistance, known as systemic acquired resistance (SAR), is an attractive target for the development of new plant protection agents. SAR induction is a multistep process that includes accumulation of pathogenesis-related (PR) proteins. The structure−activity profile of salicylates and related compounds has been evaluated using an inducible PR protein (PR-1a) and plant resistance to tobacco mosaic virus (TMV) as markers. Among the 47 selected monosubstituted and multiple-substituted salicylate derivatives tested, all 8 derivatives that induced more PR-1a protein than SA were fluorinated or chlorinated in the 3- and/or 5-position (3,5-difluorosalicylate > 3-chlorosalicylate > 5-chlorosalicylate > 3,5-dichlorosalicylate > 3-chloro-5-fluorosalicylate > 3-fluorosalicylate > 3-fluoro-5-chlorosalicylate > 3,5-dichloro-6-hydroxysalicylate > SA). In general, substitutions for or on the 2-hydroxyl group or at the 4-position of the ring reduced or eliminated PR-1a protein induction. In contrast, substitutions in positions ortho (3-position) or para (5-position) to the hydroxyl group with electron-withdrawing groups other than chlorine or fluorine decreased induction, and electron-donating groups in these positions also had a deleterious effect on PR-1a induction. PR-1a protein accumulation and reduction in TMV lesion diameter exhibited a log-linear relationship. The seven salicylate derivatives that were the most active TMV resistance inducers were all halogenated in the 3- and/or 5-position (3-chlorosalicylate > 3,5-difluorosalicylate > 3,5-dichloro-6-hydroxysalicylate > 3,5,6-trichlorosalicylate > 5-chlorosalicylate > 5-fluorosalicylate > 3,5-dichlorosalicylate > 4-fluorosalicylate > 3-fluorosalicylate > 3-chloro-5-fluorosalicylate > 4-chlorosalicylate > SA). The correlation between PR-1a protein induction and resistance to TMV confirms the value of using PR-1a induction as a screening tool for developing new plant disease control agents.

KEYWORDS: Systemic acquired resistance; salicylic acid; tobacco mosaic virus; PR-1a protein; halogenation

INTRODUCTION

Plants possess an inducible defense response known as systemic acquired resistance (SAR). SAR is defined as an increased resistance to disease in noninfected plant parts following pathogen attack (*1*). Although SAR has been known for more than 70 years (*2*), its basis is still being elucidated. The trigger for SAR is the recognition of the invading pathogen, and the result is the mounting of a resistance response. This response is often characterized by a plant-mediated hypersensitive response (HR) that may prevent the spread of a pathogen (*3*). SAR implies that a signal or signals move from pathogen-exposed to pathogen-free parts of the plant. In addition to the SAR pathway, which is salicylic acid (SA) dependent, an induced systemic resistance (ISR) pathway is also active in plants. ISR, which is independent of SA, acts through jasmonate and ethylene and may be either antagonized or complemented by the SAR pathway (*4*).

Studies with SA have shown its importance in plant−pathogen interactions. SA levels increased both locally and systemically following the HR to invading pathogens (*5*, *6*). Furthermore, exogenous SA induces the accumulation of mRNA for the pathogenesis-related (PR) proteins that are associated with SAR (*7*). Two classes of PR proteins, chitinases and glucanases, have antimicrobial properties in vitro or when overexpressed in transgenic plants (*8*). Other PR proteins such as PR-1 are of unknown function and may reduce pathogen colonization through yet undetermined mechanisms (*9*). Whether SA is the primary signal in tobacco SAR remains controversial (*10*, *11*). The necessity of SA for the expression of SAR in tobacco was shown in experiments with the salicylate hydroxylase (*nahG*) transgene, which converts salicylate into catechol.

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Overexpression of the *nahG* gene in tobacco plants blocks SA accumulation and prevents the development of SAR (*12*). Although questions remain regarding the role and mode of action of SA in SAR, most studies support the role of SA as a signal in plant disease resistance (*13*).

An increased understanding of SAR has led to the development of pesticides that induce plant defenses. For example, acibenzolar-*S*-methyl, the active ingredient in Actigard 50WG, was discovered and developed to induce disease resistance in plants (*3*). In Japan, Oryzemate, with the active ingredient probenazole, is used to control rice blast disease through SAR induction (*14*). However, more effective crop protection agents are needed. We have used a tiered approach to screen SA and salicylate derivatives, in which the primary screen quantifies the induction and accumulation of PR-1a protein, a prominent acidic protein induced during SAR, and the second tier tests the ability to increase resistance of tobacco to tobacco mosaic virus (TMV).

MATERIALS AND METHODS

Chemicals. SA, most other salicylates, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). 3-Formylsalicylate was obtained from Acros Organics (Fisher Scientific, Pittsburgh, PA). 3-Fluorosalicylic acid and 3′,5′-difluoro-2′-hydroxyacetophenone were obtained from Matrix Scientific (Columbia, SC). Crop oil concentrate (COC) used in all foliar sprays comprised 83% Orchex 796 (Exxon Co., Houston, TX) and 17% AT Plus 300F (Uniqema, New Castle, DE). The syntheses of 6-methylsalicylic acid, 3,5-difluorosalicylic acid, 3-fluoro-5-chlorosalicylic acid, 3-methoxy-5-chlorosalicylic acid, 3-chloro-5-fluorosalicylic acid, 3,5-dichloro-6-hydroxysalicylate, and 2,6-dichloroisonicotinic acid are described below.

6-Methylsalicylic Acid. Ethyl 6-methylsalicylate was hydrolyzed by heating under reflux for 2 h with 3 equiv of dilute aqueous sodium hydroxide solution. Addition of 0.5 M sulfuric acid precipitated the product in 95% yield. The analytical sample was recrystallized from dichloroethane: mp 165−167 °C with sublimation; GC-MS, *m*/*z* 152 [M^+], 134, 106, 105, 78; ESI-MS, *m*/*z* 151 [M − H]$^-$.

3,5-Difluorosalicylic Acid. 3′,5′-Difluoro-2′-hydroxyacetophenone (1.033 g, 6 mmol, 1 equiv) was dissolved in 5 mL of dioxane and converted to the sodium salt by the addition of 1.0 mL of 6 M NaOH (aq) and 5 mL of water. The resulting suspension was stirred in an ice−water bath while a solution of 4.72 g of iodine (18.6 mmol, 3.1 equiv) and 3.08 g of potassium iodide (18.6 mmol, 3.1 equiv) in 30 mL of water was added in small portions, alternating with five additional 1.0 mL portions of 6 M NaOH (aq). The addition took 1 h, and stirring was continued in the cold for an additional hour. Treatment with a little sodium bisulfite removed excess iodine. The pH of the mixture was adjusted to 9 with 5 N sulfuric acid, and the dioxane was removed as an azeotrope on a rotary evaporator. The mixture was again cooled to ice-bath temperature, and the precipitated iodoform was removed by filtration. Further acidification to pH 1.5 with sulfuric acid precipitated the product, which was collected by filtration, washed with chilled water acidified by the addition of a small amount of trifluoroacetic acid, and air-dried. Yield was 582 mg, plus another 107 mg upon concentration of the mother liquors (65% total): mp 182−184 °C with sublimation [lit., 185−186 °C (*15*)]; GC-MS, *m*/*z* 174 [M^+], 156, 128, 100.

3-Fluoro-5-chlorosalicylic Acid. 3-Fluorosalicylic acid (624 mg, 4 mmol, 1.0 equiv) was dissolved in 4 mL of dioxane to give a clear, colorless solution. Hydrochloric acid (0.70 mL of 6.0 M, 4.2 mmol, 1.05 equiv) was added, producing a two-phase mixture. Hydrogen peroxide (0.50 mL of 30%, 4.4 mmol, 1.1 equiv) was added with rapid stirring over a period of 1 min, and the reaction mixture was stirred overnight at ambient temperature and protected from light. The next day a further 0.70 mL of 6 M hydrochloric acid and 0.50 mL of 30% hydrogen peroxide were added, and stirring was continued for another 24 h. Finally, another 0.05 mL of 30% hydrogen peroxide was added, and stirring was continued overnight once more. The reaction mixture was concentrated under a stream of argon until an abundant deposit of crystals had formed, and a recrystallization was performed by heating to redissolve them. Two crops of crystals were taken for a total yield of 640 mg of colorless solid: mp 173−175 °C; GC-MS, after methylation with boron trifluoride/methanol, *m*/*z* 204/206 [M^+ for methyl ester], 173/175, 172/174, 144/146, 117/119, 116/118 and 81; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.40 (1H, dd, J = 2.58, 10.48 Hz, H-4), 7.62 (1H, dd, J = 1.88, 2.50 Hz, H-6); ^{19}F NMR (CD_3OD, 376 MHz) δ −136.01 (1F, d, J = 10.45 Hz, F-3).

3-Methoxy-5-chlorosalicylic Acid. This compound was prepared from 3-methoxysalicylic acid according to the method described above for 3-fluoro-5-chlorosalicylic acid, except that the initial addition of hydrogen peroxide was carried out in an ice−water bath. Total yield for two crops of crystals was 65%: mp 189−192 °C; ESI-MS, *m*/*z* 201 (100%), 203 (31.3%) [M − H]$^-$.

3-Chloro-5-fluorosalicylic Acid. This compound was prepared from 5-methoxysalicylic acid according to the method described above for 3-fluoro-5-chlorosalicylic acid, except that 8 equiv of HCl and 9 equiv of hydrogen peroxide were required to drive the reaction to completion: mp 216−218 °C (with sublimation) after recrystallization from dichloroethane [lit., 220−221 °C (*16*)]; ESI-MS, *m*/*z* 189 (100%), 191 (31.3%) [M − H]$^-$.

3,5-Dichloro-6-hydroxysalicylate (2,6-Dihydroxy-3,5-dichlorobenzoic Acid). This compound was prepared from 2,6-dihydroxybenzoic acid according to the method described above for 3-fluoro-5-chlorosalicylic acid except that the initial addition of hydrogen peroxide was carried out in an ice−water bath. Two crops of crystals provided a total yield of 85%: mp 210−212 °C [lit., 210−212 °C (*17*)]; ESI-MS, *m*/*z* 221 (100%), 223 (62%), 225 (11%) [M − H]$^-$.

2,6-Dichloroisonicotinic Acid. This compound was prepared by the reaction of citrazinic acid with phosphoryl chloride at 125 °C under pressure for 24 h (*18*): ESI-MS, *m*/*z* 190 (100%), 192 (58.3%), 193.9 (10.0%) [M − H]$^-$.

Plant Material. Tobacco (*Nicotiana tabacum* cv. Xanthi-nc) seed was obtained from Dr. Ilya Raskin (Rutgers University, New Brunswick, NJ). The Xanthi-nc variety carries the N-gene for resistance to TMV; thus, it responds hypersensitively to TMV. Tobacco plants were grown in Promix PGX (25 °C; 16:8 h light/dark cycle). Plants were given 1 g/L of 20−20−20 fertilizer twice a week. Plants were treated with test compounds or virus at 5−6 weeks after sowing, at which time 5−7 leaves were fully expanded.

PR-1a Protein Induction. Test compounds were dissolved in ethanol and then diluted in water to the target concentration. Treatment solutions contained 0.1% (v/v) ethanol. Treatment solutions were infused through the stomata of the abaxial surface of the youngest fully expanded leaves. The solutions were forced into the leaves by a disposable syringe without a needle at multiple locations of the leaves until they were saturated. In all studies, a positive control treatment of 1 mM SA was included to allow determination of the relative PR-1a induction at 96 h.

Proteins were harvested according to the method of Yalpani et al. (*19*). All harvests were performed 96 h after infiltration unless otherwise indicated. Briefly, treated leaves were deveined, and the remaining tissue was cut into 1 cm × 1 cm pieces and vacuum-infiltrated by immersion in ice-cold extraction buffer. The extraction buffer contained 25 mM Tris, pH 7.8, 25 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 M sucrose, 20 μM phenylmethanesulfonyl fluoride, and 150 μM 2-mercaptoethanol (*20*). Leaf tissue was blotted to remove excess buffer and placed in a spin column assembly. The intercellular fluid containing the secreted PR-1a protein was collected by centrifugation at 1000*g* for 10 min at 4 °C and stored at −80 °C prior to electrophoresis.

Protein concentrations of intercellular fluids were determined with Bradford dye reagent (Bio-Rad, Richmond, CA) using BSA as a standard. Aliquots of 10 μg of total protein/sample were analyzed by electrophoresis on precast 14% native Tris−glycine polyacrylamide gels (Invitrogen, Carlsbad, CA). Gels were stained with colloidal blue staining reagent (Invitrogen), and the band corresponding to PR-1a protein was quantified using a model GS-710 densitometer (Bio-Rad). The identity of the 14 kDa protein band that corresponds to PR-1a has been confirmed using a polyclonal antibody (*19*, *21*).

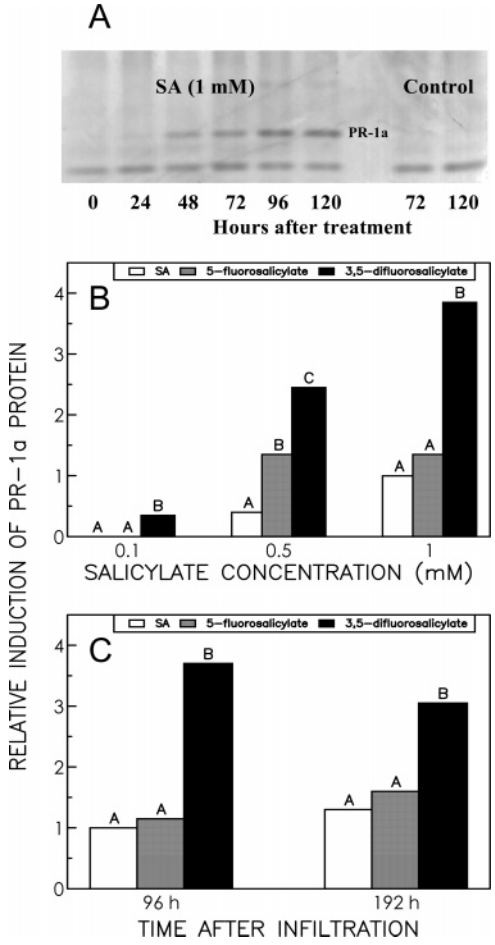

Figure 1. Time course of salicylate (SA) induction of pathogenesis-related protein (PR-1a) induction in Xanthi-nc tobacco (**A**) and effects of salicylate (SA; 5-fluorosalicylate, and 3,5-difluorosalicylate) concentration (**B**) and time after infiltration (**C**) on PR-1a induction. Relative induction of PR-1a is the amount of PR-1a protein induced by test compound (1 mM) divided by that induced by SA (1 mM) 96 h after infiltration. Means were separated by Duncan's new multiple-range test ($p = 0.05$; $n = 3$ replicate studies). Means followed by the same letter are not significantly different.

TMV Resistance. Test compounds were dissolved in ethanol and then diluted in water (treatment solutions contained 0.1% v/v ethanol). Spray solutions were amended with 0.25% COC. Plants were sprayed to drip and arrayed in a randomized complete block experimental design in a greenhouse (25 °C; 16:8 h light/dark cycle). Unless otherwise stated, one leaf per plant was inoculated 4 days after spray application with 1.0 μg of TMV (U1 strain) (*21*). Six days after inoculation, inoculated leaves were removed, and diameters of 20 necrotic lesions per leaf were measured with the aid of a dissecting microscope.

Statistical Analysis. Data were subjected to analysis of variance, and means were separated by Duncan's new multiple-range test. The relationship between relative induction of PR-1a protein and reduction in TMV lesion diameter was determined by regression analysis using PlotIT software (Scientific Programming Enterprises, Haslett, MI).

RESULTS AND DISCUSSION

PR-1a Protein Induction. Induction of PR-1a protein by 1 mM SA increased with time (**Figure 1A**; optical density × 10/mm^2 is 0, 0.05, 0.30, 0.46, 0.83, and 0.98 for 0, 24, 48, 72, 96, and 120 h, respectively). The optimal SA rate and time of PR-1a collection were initially determined to establish a consistent protocol for comparative studies. Although 0.1 mM SA did not induce consistent induction of PR-1a in tobacco, induction increased linearly with SA at 0.5 and 1 mM (**Figure 1B**). Higher rates (e.g., 2 mM SA) induced more PR-1a in a dose-dependent manner, but also produced phytotoxicity. Because phytotoxicity for 1 mM SA was limited only to the points of infiltration, this rate was used as the standard in further studies. PR-1a induction was initially measurable 24 h after infiltration with 1 mM SA (**Figure 1A**) and increased with time through 192 h. However, the most consistent response among studies was found at 96 h after infiltration. In all studies, 1 mM SA was used as a treatment and an internal standard against which all other compounds were compared. At least two plants (one leaf per plant) were treated with each compound, and the samples obtained were used to obtain PR-1a induction data. The densities of the PR-1a bands obtained with the 1 mM SA samples were averaged and normalized to 1.0. The densities of the PR-1a bands for each test compound were averaged, and their induction is expressed relative to 1 mM SA. All test compounds were assayed in at least two independent experiments, and the final induction number is an average of these studies.

Among the 36 monosubstituted salicylates tested (**Table 1**), 3 had substantially greater PR-1a induction than SA (3-chlorosalicylate, 3-fluorosalicylate, and 5-fluorosalicylate with 167, 113, and 60% greater induction, respectively). Within a family of derivatives, 3- or 5-position substitutions were more active than 4- or 6-position substitutions (e.g., 3-fluorosalicylate > 5-fluorosalicylate > 4-fluorosalicylate > 6-fluorosalicylate). With the exception of 3-aminosalicylate, 3-methoxysalicylate, and 5-cyanosalicylate (relative PR-1a inductions of 0.54, 0.49, and 0.85, respectively), the only 3- or 5-substituted salicylate derivatives showing substantial PR-1a induction were the small halogen-substituted salicylate derivatives. The 5-substituted halosalicylates show decreasing activity with the increase in atomic weight and steric bulk of the substituent group (i.e., activity of 5-fluorosalicylate > 5-chlorosalicylate > 5-bromosalicylate > 5-iodosalicylate). Substitution with other strong electron-withdrawing groups with larger steric requirements, such as nitro, cyano, or aldehyde, resulted in less activity than with the smaller halogens at comparable positions.

Among the 11 di- and trisubstituted salicylates tested (**Table 2**), 5 had substantially greater PR-1a induction than SA (3,5-difluorosalicylate, 3,5-dichlorosalicylate, 3-chloro-5-fluorosalicylate, 3-fluoro-5-chlorosalicylate, and 3,5-dichloro-6-hydroxysalicylate with 283, 81, 70, 46, and 41% greater induction, respectively). Whereas fluorination and/or chlorination in both the 3- and 5-positions improved activity, PR-1a induction was greatly reduced by the substitution of an additional halogen to the 6-position (i.e., 3,5,6-trichlorosalicylate) or eliminated by the substitution of bulkier groups, even if they are electron-withdrawing, at the 3- and 5-positions (e.g., 3,5-dinitrosalicylate).

From the PR-1a-inducing activity of the monosubstituted and multiply substituted salicylates, we can conclude that enhanced activity is observed only when specific conditions are met: (1) Enhanced activity is limited to substitution at the 3- and 5-positions. Substitutions at positions other than 3 and 5 of the ring result in decreased activity. (2) Substituents must be electron-withdrawing to enhance activity. (3) Substituents may contain no more than one atom and have a van der Waals radius no greater than that of chlorine. Also, position 3 is more tolerant of steric bulk than position 5.

The dose response of SA compared to two fluorinated salicylates is shown in **Figure 1B**. At the lowest rate tested (0.1 mM), 3,5-difluorosalicylate induced a measurable amount of PR-1a, whereas neither SA nor 5-fluorosalicylate induced at that rate. At either 0.5 or 1.0 mM, both 5-fluorosalicylate and

Table 1. Induction of Pathogenesis-Related Protein (PR-1a) in Xanthi-nc Tobacco by Salicylates with a Single Modification or Substitution

compound[a]	substituent	relative induction of PR-1a[b]
control		0.00
SA		1.00
position 1 substitution		
salicylamide	$CONH_2$	0.00
position 2 substitution		
acetylsalicylate*	acetoxy	1.10
benzoate	H	0.00
2-fluorobenzoate	F	0.06
thiosalicylate	SH	0.59
position 3 substitution		
3-aminosalicylate	NH_2	0.54
3-chlorosalicylate	Cl	2.67
3-fluorosalicylate	F	2.13
3-formylsalicylate	CHO	0.06
3-hydroxylsalicylate	OH	0.00
3-isopropylsalicylate	isopropyl	0.00
3-methoxysalicylate	OCH_3	0.49
3-methylsalicylate	CH_3	0.22
3-nitrosalicylate	NO_2	0.27
3-phenylsalicylate	phenyl	0.00
position 4 substitution		
4-aminosalicylate	NH_2	0.08
4-chlorosalicylate*	Cl	0.32
4-fluorosalicylate	F	0.83
4-hydroxylsalicylate	OH	0.00
4-methoxysalicylate	OCH_3	0.17
4-methylsalicylate	CH_3	0.05
position 5 substitution		
5-aminosalicylate	NH_2	0.00
5-bromosalicylate	Br	0.41
5-chlorosalicylate*	Cl	1.07
5-cyanosalicylate	CN	0.85
5-fluorosalicylate	F	1.60
5-formylsalicylate	CHO	0.00
5-hydroxylsalicylate	OH	0.00
5-iodosalicylate	I	0.00
5-methoxysalicylate	OCH_3	0.12
5-methylsalicylate	CH_3	0.00
5-nitrosalicylate	NO_2	0.15
position 6 substitution		
6-fluorosalicylate	F	0.29
6-hydroxylsalicylate	OH	0.86
6-methoxysalicylate	OCH_3	0.31
6-methylsalicylate	CH_3	0.16

[a] Asterisk denotes known SAR inducer [Conrath et al. (*28*)]. [b] Amount of PR-1a protein induced by test compound (1 mM) divided by that induced by salicylate (1 mM; SA) 96 h after infiltration.

3,5-difluorosalicylate acid induced significantly more PR-1a protein than SA at the same concentrations. This enhanced accumulation of PR-1a protein extended through 192 h after infiltration (**Figure 1C**).

TMV Resistance Induction. Among the 16 methoxy-, chloro-, and fluorosalicylates tested, induction of resistance to TMV generally paralleled induction of PR-1a (**Figure 2**). The relationship between relative induction of PR-1a protein and reduction in TMV lesion diameter was log-linear:

$$\text{TMV lesion diameter (\% reduction)} = 35.5 + 23.4 \times \log(\text{relative induction of PR-1a protein}); \quad r^2 = 0.54$$

Except for 6-fluorosalicylate, all fluoro- and chlorosalicylates tested were more active TMV resistance inducers than SA (3-chlorosalicylate > 3,5-difluorosalicylate > 3,5-dichloro-6-hydroxysalicylate > 3,5,6-trichlorosalicylate > 5-chlorosalicylate > 5-fluorosalicylate > 3,5-dichlorosalicylate > 4-fluorosalicylate > 3-fluorosalicylate > 3-chloro-5-fluorosalicylate > 4-chlorosalicylate > SA). All four methoxylated salicylate derivatives were less active than SA.

Table 2. Induction of Pathogenesis-Related Protein (PR-1a) in Xanthi-nc Tobacco by Salicylates with Multiple Substitutions

compound[a]	relative induction of PR-1a[b]
control	0.00
SA	1.00
dihalogen substitutions	
3,5-dibromosalicylate	0.25
3,5-dichlorosalicylate*	1.81
3,5-difluorosalicylate	3.83
mixed dihalogen substitutions	
3-chloro-5-fluorosalicylate	1.70
3-fluoro-5-chlorosalicylate	1.46
other multiple substitutions	
3,5-dichloro-6-hydroxysalicylate	1.41
2,6-difluorobenzoic acid	0.00
5-(2,4-difluorophenyl)salicylic acid	0.00
3,5-dinitrosalicylate	0.00
3-methoxy-5-chlorosalicylate	0.15
3,5,6-trichlorosalicylate	0.25
2,3,6-trifluorobenzoic acid	0.02

[a] Asterisk denotes known SAR inducer [Conrath et al. (*28*)]. [b] Amount of PR-1a protein induced by test compound (1 mM) divided by that induced by salicylate (1 mM; SA) 96 h after infiltration.

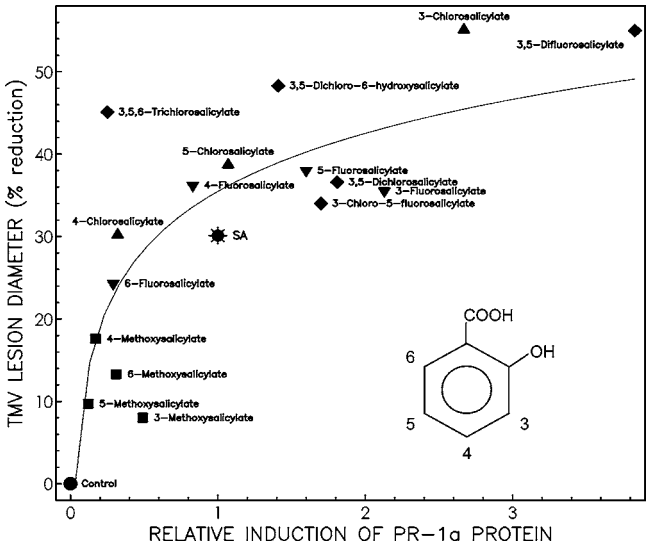

Figure 2. Relationship between relative induction of pathogenesis-related protein (PR-1a) and percent reduction in tobacco mosaic virus (TMV) lesion diameter in Xanthi-nc tobacco for methoxysalicylates (square), chlorosalicylates (triangle), fluorosalicylates (inverted triangle), and salicylate (SA; asterisk over circle) with multiple substitutions (diamond). Relative induction of PR-1a is the amount of PR-1a protein induced by test compound (1 mM) divided by that induced by SA (1 mM) 96 h after infiltration. TMV lesion diameter was determined for 1 mM of test compound. Line represents TMV lesion diameter (percent reduction) = 35.5 + 23.4 × log(relative induction of PR-1a protein); $r^2 = 0.54$. Structure of SA is in inset.

At 48 h after treatment, 1 mM 3,5-difluorosalicylate reduced TMV lesion size slightly more than an equal molar concentration of SA, and almost as well as 5 mM SA (**Figure 3**). At 96 h after inoculation, 1 mM 3,5-difluorosalicylate was more effective at reducing TMV lesions than 5 mM SA. This indicates that 3,5-difluorosalicylate, which is the best PR-1a inducer among

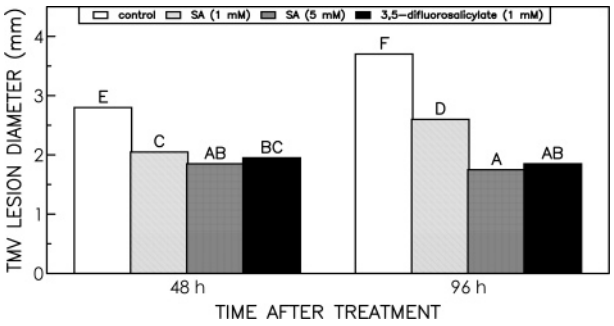

Figure 3. Tobacco mosaic virus (TMV) lesion diameter reduction by salicylate (SA; 1 and 5 mM) and 3,5-difluorosalicylate (1 mM). TMV was inoculated either 48 or 96 h after spray applications, and lesions were measured 6 days later. Means were separated by Duncan's new multiple-range test ($p = 0.10$; $n = 4$ plants). Means with the same letter are not statistically different.

the salicylates (**Tables 1** and **2**), is more persistent and effective at a lower rate than SA.

The effect of structure modification on salicylate activity has been examined previously in other systems. These include studies on the reduction of inflammation (*22*, *23*), inhibition of the mammalian endothelin ETA receptor (*24*), heat production in *Arum* lily (*25*), NO_x emission by soybean (*26*), glucosyltransferase activity in TMV infected tobacco (*27*), TMV lesions size reduction, PR-1 protein induction, catalase activity, and SA binding inhibition (*28*), and herbicide modulation (*29*, *30*). The structure−activity relationship of salicylates is dependent on the assay system. For example, 5-(2,4-difluorophenyl)-salicylic acid (diflunisal) is a very effective anti-inflammatory drug (*22*), but was inactive as an inducer of PR-1a proteins (**Table 2**). In contrast, 3-, 4-, and 5-substituted chlorosalicylates and 5-fluorosalicylate were active NO_x inducers (*26*) and also induced PR-1a and TMV resistance in our studies. Moreover, the halogenated salicylates were among the most active potentiators of atrazine, even through atrazine potentiation is independent of SAR (*30*). The protection from the herbicide paraquat also demonstrates the superior biological activity of the halogenated salicylates over that of other derivatives (*29*).

In summary, we have shown that the induction of tobacco PR-1a is of value in predicting the ability of substituted salicylates and related compounds to induce disease resistance in plants. Among the compounds tested, we have determined that only fluorinated or chlorinated salicylates in the 3- and/or 5-ring position were more active than SA at inducing PR-1a protein. We have also demonstrated that the ability of a compound to induce PR-1a correlates with its ability to induce resistance of tobacco to TMV.

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