12. Our data were taken using a Fourier transform spectrometer. The spectrometer was fitted with a CaF_2 beam splitter, a tungsten source, and an InSb detector. Data were taken between 1 and 5 μm (2000 and 10000 cm^{-1}) and spanned a temperature range of 35 to 60 K. The spectral resolution was 5×10^{-4} μm at 2.15 μm (1 cm^{-1}). Our integration period was 4 min. Backgrounds were taken with the same integration period. The N_2 from which our samples were formed is of research quality (99.9995% pure). A sample of solid N_2 was held in a cell attached to a cold finger, cooled by a closed-cycle He refrigerator. The temperature controller used in the experiments is capable of millikelvin resolution and is able to hold a sample at a constant temperature (± 0.02 K) for several hours.
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15. The continuum absorption of N_2 is <1% in this wavelength region, which makes an accurate measurement of this quantity extremely difficult. Because it is the relative depths of the bands that are the important quantity, we chose to normalize the transmittance data to 1.0 in the continuum, providing a common reference level from which to quote results.
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Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance

Thomas Gaffney, Leslie Friedrich, Bernard Vernooij, David Negrotto, Gordon Nye, Scott Uknes, Eric Ward, Helmut Kessmann, John Ryals*

It has been proposed that salicylic acid acts as an endogenous signal responsible for inducing systemic acquired resistance in plants. The contribution of salicylic acid to systemic acquired resistance was investigated in transgenic tobacco plants harboring a bacterial gene encoding salicylate hydroxylase, which converts salicylic acid to catechol. Transgenic plants that express salicylate hydroxylase accumulated little or no salicylic acid and were defective in their ability to induce acquired resistance against tobacco mosaic virus. Thus, salicylic acid is essential for the development of systemic acquired resistance in tobacco.

Infection of plants with a necrotizing pathogen can enhance the plants' resistance to subsequent infections by a variety of bacterial, fungal, and viral pathogens (*1*). This physiological immunity, also known as systemic acquired resistance (SAR), is an integral and important component of a plant's defense against disease. Among the more interesting aspects of acquired resistance are that the resistance extends to plant tissues distant from the initial infection site, persists for weeks to months after the initial infection, and protects against a broad spectrum of plant pathogens (*1*). These characteristics make SAR a promising target for the discovery of therapeutics to control plant diseases (*2*).

In tobacco, cucumber, and *Arabidopsis*, SAR is strongly correlated with the coordinate expression of a set of genes (SAR genes) encoding proteins that include the "pathogenesis-related" (PR) proteins (*3–5*). How these proteins contribute to resistance is unknown, although several function as antibiotics when tested against plant pathogens in vitro (*6*). In addition, one of the SAR genes, PR-1a, confers tolerance to two different oomycete pathogens when it is expressed in transgenic tobacco (*7*). Thus, these proteins may be causally involved in maintaining the disease-resistant state.

Although the phenomenon of SAR has been known for almost a century and has been described in many reports (*1*), the way in which the resistant state is induced is still poorly understood. After pathogen infection, but before the onset of resistance, salicylic acid (SA) accumulates in the phloem of cucumber and tobacco plants (*8, 9*). The exogenous application of SA to tobacco leaves mimics the pathogen-induced SAR response by inducing the same set of SAR genes, as well as resistance, in treated tissue (*3, 4–10*). Consequently, it has been postulated that SA serves as an endogenous signal molecule required for SAR induction (*3, 8, 9*).

If an increase in endogenous SA is required to establish acquired resistance, then a barrier to SA accumulation should block the development of SAR. Salicylate hydroxylase (E.C. 1.14.13.1), encoded by the *nahG* gene of *Pseudomonas putida*, is a flavoprotein that catalyzes the decarboxylative hydroxylation of salicylate, converting it to catechol (Fig. 1) (*11*). A DNA fragment containing the *nahG* coding sequence from *P. putida* PpG7 (*12*) was subcloned from pSR20 (*13*) into an expression vector in which transcription is controlled by the enhanced 35S cauliflower mosaic virus promoter and terminated by the *tml* 3′ terminator (*13*). We transformed the final construct into tobacco using *Agrobacterium* (*13*). Independently transformed plants were screened for *nahG* mRNA accumulation, and several transformants were selected for further analysis. We allowed these plants to self-pollinate for two generations and then identified lines homozygous for the selectable marker by screening for antibiotic resistance.

To determine the effect of *nahG* gene expression on the accumulation of SA, we inoculated three lower leaves of plants from each of the lines with tobacco mosaic virus (TMV). After 7 days, when lesions had formed on the infected leaves, we harvested leaf tissue and assayed for *nahG* mRNA (*14*), salicylate hydroxylase protein (*15*), and SA (*16*). The nontransformed control line infected by TMV accumulated ~6000 ng of SA per gram of tissue, representing a 185-fold increase relative to plants treated with medium but no virus (Fig. 2), a result consistent with previous findings (*8, 17,*

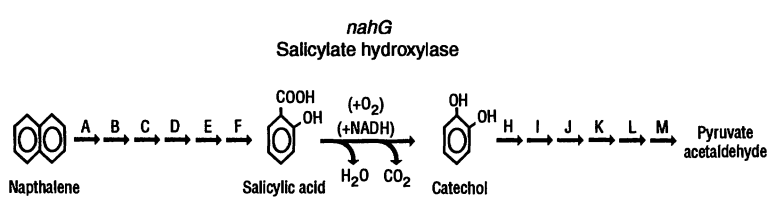

Fig. 1. Function of *nahG*. Biochemical degradation of naphthalene by *P. putida* strain PpG7 occurs in 13 steps encoded by the bacterial *nah* genes (*12*). The letters A to M represent the enzymes encoded by the genes *nahA* to *nahM*. The *nahG* gene encodes salicylate hydroxylase that uses NADH (reduced form of nicotinamide adenine dinucleotide) as a cofactor to decarboxylate and hydroxylate SA to catechol.

T. Gaffney, L. Friedrich, B. Vernooij, D. Negrotto, G. Nye, S. Uknes, E. Ward, J. Ryals, Agricultural Biotechnology Research Unit, Ciba-Geigy Corporation, Post Office Box 12257, Research Triangle Park, NC 27709. H. Kessmann, Plant Protection, Ciba-Geigy Limited, CH4002 Basel, Switzerland.

*To whom correspondence should be addressed.

18). Transgenic plants (lines NahG-3, NahG-8, and NahG-10) that accumulated substantial amounts of *nahG* mRNA and salicylate hydroxylase averaged only a two- to threefold increase of SA after TMV inoculation (Fig. 2 and Table 1). Plants that expressed intermediate amounts of mRNA and salicylate hydroxylase (lines NahG-1 and NahG-2) had moderate increases in SA accumulation (Fig. 2 and Table 1). Plants from the NahG-9 line did not contain detectable *nahG* mRNA or salicylate hydroxylase (Fig. 2) and accumulated SA to concentrations indistinguishable from the nontransformed control. Therefore, expression of *nahG* mRNA and salicylate hydroxylase in transgenic plants significantly blocked SA accumulation in TMV-treated plants.

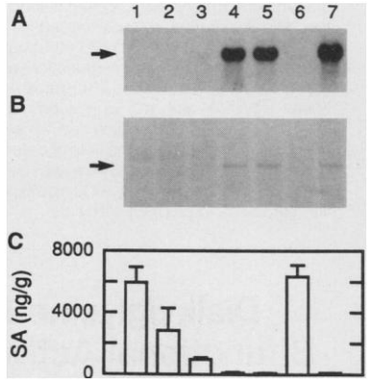

Fig. 2. Molecular analysis of transgenic tobacco plants containing the engineered *nahG* coding sequence. The sample in lane 1 is from nontransgenic *xanthi*.nc plants. Lanes 2 through 7 represent the transgenic lines NahG-1, NahG-2, NahG-3, NahG-8, NahG-9, and NahG-10, respectively. (**A**) Northern blot analysis of RNA extracted from leaf tissue 7 days after inoculation with TMV (*14*). The blots were probed with a [^{32}P]DNA fragment containing the salicylate hydroxylase coding sequence. The arrow indicates the position where full-length *nahG* mRNA should run. (**B**) Western blot analysis of protein extracted from leaf tissue of TMV-inoculated plants (*15*). The blots were reacted with antiserum raised against recombinant salicylate hydroxylase. A standard of purified salicylate hydroxylase was run and stained in this gel. The arrow indicates the position of the standard. (**C**) Amounts of SA extracted from leaf tissue of the TMV-inoculated plants (*16*), recorded as nanograms of salicylic acid per gram of leaf tissue, mean ± SD.

We determined the effect of reduced SA accumulation on SAR by exposing the TMV-infected plants to a subsequent dose of virus on their upper leaves. Lesion size 5 days after the challenge was inversely proportional to the amount of SA accumulated as a result of the primary TMV infection (Fig. 3 and Table 1). These results demonstrate that the accumulation of SA is required for the establishment of SAR.

An unexpected result in this experiment was the increase of primary lesion size in plants expressing moderate or high amounts of salicylate hydroxylase. The TMV lesions were significantly larger (on average, 23% larger) on either mock-inoculated (Table 1) or nontreated plants (*19*) compared with nontransgenic controls. This suggests a role for SA not only in SAR but also in the primary resistance response to pathogen infection.

White (*10*) initially showed that the treatment of plants with SA, benzoic acid, and aspirin could reduce the size of TMV lesions. In an extension of these results, van Loon and Antoniw (*3*) demonstrated that SA could induce accumulation of the PR proteins and that this accumulation correlated with the TMV resistance. The latter investigators suggested that SA could conceivably be a signal for SAR in plants.

The most plausible synthetic pathway for SA in higher plants branches from phenylpropanoid biosynthesis after the synthesis of *trans*-cinnamic acid. At this point, cinnamic acid is either oxidized to benzoic acid and then ortho-hydroxylated to SA or first hydroxylated and then oxidized to SA (*20*). Synthesis of SA from the phenylpropanoid pathway would provide a compelling model to link SA and SAR to the primary responses of the plant to pathogen attack (*4*). However, the proposed pathways for SA biosynthesis are based on suggestive evidence, and the pathogen-induced synthesis of SA may be through an alternative pathway such as one involving *iso*-chorismate, as has been demonstrated for *Mycobacterium smegmatis* (*21*).

Although our data indicate that increased endogenous SA is required for the establishment of SAR, they do not address whether SA is the systemically transmitted signal. Cucumber leaves infected with *Pseudomonas syringae* can be excised 4 hours after infection, before a significant increase in SA, without affecting either the accumulation of SA or the SAR response in the intact, but now uninfected, plant (*22*). Thus, another as yet unidentified signal may also be important for systemic responses in the induction of SAR.

Table 1. Salicylic acid and TMV lesion size in buffer-pretreated and TMV-pretreated tobacco plants. Salicylic acid is expressed as nanograms of SA per gram of extracted tissue. The values are the means ± SD after correction for recovery during the extraction (57.1%) and are based on triplicate assays, except for the NahG-1 mock-inoculated sample which was based on a duplicate assay. The sizes of TMV lesions are given in millimeters. The numbers are the mean ± SD. We measured 90 lesions from three to five plants per line. The data were statistically analyzed by analysis of variance (ANOVA II) and then by a Tukey-Kramer test. Within each treatment category, statistically equivalent groups ($P \geq 0.05$) are indicated by letters a through d.

Line	Salicylic acid (ng/g)	Fold induction*	TMV lesion size ± SD (mm)	
			Buffer pretreated	TMV pretreated
xanthi	5937 ± 1011	185	3.5 ± 0.4 (d)	1.3 ± 0.5 (d)
NahG-1	2824 ± 1461	79	4.1 ± 0.4 (b)	2.7 ± 0.6 (c)
NahG-2	979 ± 113	25	4.4 ± 0.4 (a)	3.8 ± 0.5 (b)
NahG-3	107 ± 45	3	4.4 ± 0.4 (a)	4.0 ± 0.5 (b)
NahG-8	81 ± 22	2	4.2 ± 0.4 (b)	4.1 ± 0.7 (b)
NahG-9	6334 ± 765	179	3.8 ± 0.4 (c)	1.3 ± 0.7 (d)
NahG-10	112 ± 4	3	4.5 ± 0.4 (a)	4.2 ± 0.8 (a)

*Expressed as amount of SA in TMV-inoculated tissue, divided by amount of SA in mock-inoculated tissue.

Fig. 3. Typical SAR response observed in transgenic plants containing the *nahG* coding sequence. Seven days after initial TMV infection, the upper, uninfected leaves were challenge-inoculated. After six more days, challenge-inoculated leaves were excised and photographed. The leaf on the left is from the NahG-9 line that has no detectable *nahG* mRNA or salicylate hydroxylase and accumulates wild-type levels of SA after TMV infection. The middle leaf is from the NahG-1 line that expresses intermediate levels of mRNA and protein and accumulates intermediate levels of SA. The leaf on the right is from the NahG-10 line that expresses high levels of mRNA and protein and accumulates very little SA after TMV inoculation.

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13. The 1.5-kb Ssp I to Hpa I fragment from plasmid pSR20 (a subclone of NAH7) was ligated to Eco RI linkers, digested with Eco RI, and subcloned into pCGN1761 [L. Comai, P. Moran, D. Maslyar, *Plant Mol. Biol.* **15**, 373 (1990)] behind an enhanced 35S promoter from cauliflower mosaic virus [K. McBride and K. Summerfelt, *ibid.* **14**, 269 (1990)]. We digested the resulting plasmid with Xba I to release a 4.8-kb fragment containing the promoter, the *nahG* coding sequence, and a *tml* 3′ terminator sequence. The Xba I fragment was ligated into the binary vector pCIB200 and the resulting plasmid was transformed into *Agrobacterium tumefaciens* CIB542 [S. Uknes *et al.*, *Plant Cell* **5**, 159 (1993)]. *Nicotiana tabacum* cv. xanthi.nc leaf disks were transformed essentially as described [R. B. Horsch *et al.*, *Science* **227**, 1229 (1985)] with the *Agrobacterium* strain containing the *nahG* construct.
14. *Nicotiana tabacum* cv. xanthi.nc and transgenic *nahG* tobacco plants were inoculated with TMV or mock-inoculated with buffer and carborundum as described [G. Payne *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 98 (1990)]. We treated a total of eight plants of each line. Tissue from mock-inoculated (*19*) or TMV-inoculated plants was harvested and pooled 7 days after inoculation. We extracted RNA as described [M. Lagrimini, W. Burkhart, M. Moyer, S. Rothstein, *ibid.* **84**, 7542 (1987)] and analyzed it by RNA blot hybridization [F. Ausubel *et al.*, *Current Protocols in Molecular Biology*, (Wiley, New York, 1987)]. We probed the gel blots with a ^{32}P-labeled DNA fragment containing the *nahG* coding sequence. Seven days after infection secondary, uninfected leaves were inoculated with TMV. Leaves were photographed 6 days after this inoculation.
15. We lyophilized and extracted the harvested tissue with sample buffer (125 mM tris, 10% β-mercaptoethanol, 4% SDS, 20% glycerol, 0.002% bromophenol blue) using 45 mg of tissue per milliliter of buffer. The protein extracts were separated by SDS–polyacrylamide gel electrophoresis on a 10 to 20% tris-tricine gel (Novex). The polypeptides were then electroblotted [H. Towbin, T. Staehlin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)] to nitrocellulose and immunostained as described [L. H. Pratt, D. W. McCurdy, Y. Shimazaki, M. M. Cordonnier, *Mod. Methods Plant Anal. New Ser.* **4**, 51 (1986)]. Antiserum was prepared by Berkeley Antibody Company (San Francisco) from rabbits injected with salicylate hydroxylase isolated from *Escherichia coli* containing the *nahG* coding sequence in the pGEX-2T expression plasmid (Pharmacia LKB Biotechnology).
16. Salicylic acid was extracted from leaf tissue essentially as described (*17*), except the samples were not allowed to overdry, and the final dried samples were resuspended in 500 μl of 20% methanol. We injected 10 to 100 μl of this suspension onto a Dynamax 60A, 8-μm, C-18 (4.6 mm by 25 cm) column with a guard column (Rainin Instruments, Emeryville, CA) maintained at 40°C. Isocratic separation was performed at 1 ml/min using 20% (v/v) methanol in 20 mM sodium acetate, pH 5.0. Fluorescence detection was done with a Model 980 detector (ABI/Kratos Analytical, Foster City, CA) with a 5 μl flow cell, deuterium lamp with a 295-nm excitation setting, and a 370-nm cutoff emission filter. The limit of detection was 500 pg of SA in 50 μl. Quantitation was determined versus a linear range (10 to 1000 ng/ml) of calibration standards for sodium salicylate.
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23. We thank S. Dincher for initial experiments showing that catechol was not an inducer of SAR; S. Dincher, G. Crawford, S. Sips, and P. VanBourgondien for horticultural support; D. Maddox for assistance on the statistical analysis; M. Schell for providing pSR20; M.-D. Chilton, B. Miflin, B. Lee, T. Brears, D. Alexander, T. Delaney, S. Foley, and K. Lawton for critically reading the manuscript; and B. Gibbons, F. Beard, and D. Hill for providing T.G. with the necessary inspiration to apply *Pseudomonas* genetics toward a better understanding of SAR.

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Dialkylglycine Decarboxylase Structure: Bifunctional Active Site and Alkali Metal Sites

Michael D. Toney,* Erhard Hohenester, Sandra W. Cowan,† Johan N. Jansonius‡

The structure of the bifunctional, pyridoxal phosphate–dependent enzyme dialkylglycine decarboxylase was determined to 2.1–angstrom resolution. Model building suggests that a single cleavage site catalyzes both decarboxylation and transamination by maximizing stereoelectronic advantages and providing electrostatic and general base catalysis. The enzyme contains two binding sites for alkali metal ions. One is located near the active site and accounts for the dependence of activity on potassium ions. The other is located at the carboxyl terminus of an α helix. These sites help show how proteins can specifically bind alkali metals and how these ions can exert functional effects.

Pyridoxal-5′-phosphate (PLP) is the most versatile of all the known coenzymes. Its preponderance in amino acid metabolism arises from its ability to labilize all four bonds to the α carbon and, less commonly, those to the β and γ carbons. The broad-ranging power of PLP is explained by the general mechanism proposed by Metzler *et al.* and Braunstein and Shemyakin (*1*), which invokes a central, carbanionic quinonoid intermediate.

Dialkylglycine decarboxylase (DGD) from *Pseudomonas cepacia* is an unusual PLP-dependent enzyme that catalyzes both decarboxylation and transamination in its normal catalytic cycle (*2*). In the first half-reaction, dialkylglycines such as α-methylalanine are oxidatively decarboxylated to give CO_2 and a ketone, with amino group transfer to the coenzyme. The second half-reaction is a classical transamination with pyruvate as substrate. This intriguing ability to catalyze rapidly both decarboxylation and transamination provided the impetus for the present study.

The DGD is a tetramer, built up as a dimer of dimers (Fig. 1 and Tables 1 and 2) (*3*). The fold of DGD is related to those of aspartate aminotransferase (AAT) (*4*), ω-amino acid aminotransferase (*5*), and tyrosine phenol-lyase (*6*) and likely represents a general template for a large family of PLP-dependent enzymes. Each DGD monomer is composed of two distinct domains and an NH_2-terminal segment. The large domain contains the PLP binding site and consists of a central, seven-stranded β sheet surrounded by eight α helices in a typical αβ fold. The small domain comprises a four-stranded β sheet with three α helices packed against the face opposite the large domain. The NH_2-terminal helix is spatially removed from the bulk of the subunit and forms a clamp to the neighboring subunit. A small, three-stranded β sheet completes the NH_2-terminal segment.

Department of Structural Biology, Biocenter of the University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland.

*Present address: Department of Chemistry, University of California, Berkeley, CA 94720.
†Present address: Ciba-Geigy AG, Biotechnology, K-681.5.03, P.O. Box, 4002 Basel, Switzerland.
‡To whom correspondence should be addressed.