Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4202–4205, May 1995
Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Signal transduction in systemic acquired resistance

JOHN RYALS*†, KAY A. LAWTON*, TERRENCE P. DELANEY*, LESLIE FRIEDRICH*, HELMUT KESSMANN‡, URS NEUENSCHWANDER*, SCOTT UKNES*, BERNARD VERNOOIJ*, AND KRIS WEYMANN*

*Department of Molecular Genetics, Biotechnology Research Unit, CIBA–Geigy Corporation, P.O. Box 12257, Research Triangle Park, NC 27709-2257; and ‡Plant Protection, CIBA–Geigy Limited, CH-4002 Basel, Switzerland

ABSTRACT **Systemic acquired resistance (SAR) is an important component of plant defense against pathogen infection. Accumulation of salicylic acid (SA) is required for the induction of SAR. However, SA is apparently not the translocated signal but is involved in transducing the signal in target tissues. Interestingly, SA accumulation is not required for production and release of the systemic signal. In addition to playing a pivotal role in SAR signal transduction, SA is important in modulating plant susceptibility to pathogen infection and genetic resistance to disease. It has been proposed that SA inhibition of catalase results in H_2O_2 accumulation and that therefore H_2O_2 serves as a second messenger in SAR signaling. We find no accumulation of H_2O_2 in tissues expressing SAR; thus the role of H_2O_2 in SAR signaling is questionable.**

In many plant species, infection by a necrotizing pathogen leads to broad-spectrum, long-lasting, systemic resistance to subsequent infection. This response has been recognized since as early as 1901 and has been called systemic acquired resistance (SAR) (for reviews see refs. 1–4). Considerable attention has recently been directed at determining the molecular basis for SAR. An understanding of the mechanisms for induction and maintenance of resistance could lead to the development of both novel plant protection chemicals and genetically engineered plants with enhanced disease resistance.

The most thoroughly characterized example of SAR is the response in tobacco (5). Tobacco mosaic virus (TMV) inoculation of local lesion hosts leads to enhanced resistance against subsequent infections with either TMV, *Cercospora nicotianae, Phytophthora parasitica, Peronospora tabacina*, or *Pseudomonas syringae* pv. *tabaci* (6). Concommitant with the onset of SAR, a group of at least nine gene families is coordinately expressed at high levels in uninfected (systemic) leaves (5). These SAR genes include the acidic isoforms of the pathogenesis-related (PR) proteins from tobacco (for recent reviews see refs. 7–10). Along with being associated with the acquired resistance, the proteins encoded by the SAR genes appear to play a causal role in the establishment of the heightened resistant state. For example, expression of PR-1a in transgenic tobacco can lead to significant resistance against both *Peronospora tabacina* and *Phytophthora parasitica* (11).

Salicylic acid (SA) has been shown to accumulate to high levels following infection of tobacco with TMV (12) and cucumber with either tobacco necrosis virus or *Colletotrichum lagenarium* (13). Because SA could be recovered from phloem extracts and because SA treatment of either tobacco or cucumber leaves could induce both resistance to pathogens and SAR gene expression, it was proposed that SA could serve as an endogenous SAR signal molecule (5, 12, 13). In recent years, a strong correlation between SA levels and the accumulation of PR-1 protein has been demonstrated (14).

In this paper, we will review certain experiments that address the role of SA in the signal transduction pathway leading to SAR. We will also report recent findings that show that SA is required not only for induction of the SAR pathway but also for genetically determined disease resistance. Finally, we will discuss recent findings concerning the role of SA-dependent effects of H_2O_2 in the induction of SAR and SAR gene expression.

Role of SA in SAR Signal Transduction

The bacterial enzyme salicylate hydroxylase removes the carboxyl group from SA and replaces it with a hydroxy group, thus converting SA to catechol in a very specific reaction that utilizes NADH as a cofactor (15). This protein is encoded by the *nahG* gene of *Pseudomonas putida* (16, 17). We subcloned the *nahG* coding sequence behind the cauliflower mosaic virus 35S promoter and transformed tobacco with this construct via *Agrobacterium tumefaciens* (18). Transgenic, homozygous lines were established and analyzed for *nahG* mRNA and salicylate hydroxylase protein accumulation. Lines that expressed high levels of the mRNA and protein were unable to accumulate significant levels of SA following TMV infection (Table 1). These lines were also shown to be incapable of inducing SAR (Table 1). We conclude from these experiments that SA accumulation is required for SAR.

Although the experiments described above strongly suggest that SA is required for SAR, they do not address whether SA is the translocated signal. To answer this question, we designed experiments (19) based on the observations that the signal for SAR could pass through a graft junction (20, 21). Wild-type Xanthi-nc tobacco and NahG tobacco plants were grafted as shown in Fig. 1. In control experiments, Xanthi-nc scions were grafted onto Xanthi-nc rootstocks and NahG scions were grafted onto NahG rootstocks (Fig. 1*A*). TMV inoculation of Xanthi-nc rootstocks resulted in protection against *C. nicotianae* and TMV in scion leaves, demonstrating that the signal could pass through the graft junction. TMV inoculation of NahG rootstocks did not result in SAR, as expected. Reciprocal grafts between Xanthi-nc and NahG (Fig. 1*B*) were also tested for SAR. Interestingly, when NahG rootstock leaves were inoculated with TMV, Xanthi-nc scion leaves exhibited SAR (Table 2). Furthermore, TMV inoculation of Xanthi-nc rootstocks did not lead to SAR in NahG scions (Table 2). Thus,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SAR, systemic acquired resistance; SA, salicylic acid; TMV, tobacco mosaic virus; PR, pathogenesis-related.

†To whom reprint requests should be addressed.

Table 1. Characterization of transgenic *nahG*-expressing tobacco lines

Line	mRNA	Protein	SA*	SAR†
Xanthi-nc	−	−	5937 ± 1011	++
NahG-1	−	−	2824 ± 1461	+
NahG-2	−/+	−/+	979 ± 113	−
NahG-3	++	++	107 ± 45	−
NahG-8	++	++	81 ± 22	−
NahG-9	−	−	6334 ± 765	++
NahG-10	++	++	112 ± 4	−

*Mean ± SD.
†Seven days after treatment of three lower (primary) leaves with buffer or TMV, uninfected (secondary) leaves were assayed for SAR by challenge inoculation with TMV.

it appears that SA is not the translocated signal for SAR and that the systemic signal for SAR can be released without accumulation of significant concentrations of SA. However, SA accumulation is required to transduce the signal in target tissues.

nahG Expression Causes Enhanced Susceptibility to Pathogens

When NahG tobacco plants were inoculated with TMV we noticed that the lesions grew much larger than on Xanthi-nc controls (22, 23). To further investigate this result, we inoculated plants with TMV and measured the expansion of lesions over a period of 10 days. The lesions were visible on both Xanthi-nc and NahG plants beginning at 3 days after inoculation. However, over the course of the next 7 days, the lesions on Xanthi-nc plants increased in area at a much slower rate than those of NahG plants. After 10 days, lesions on Xanthi-nc plants reached an average of 25 mm^2 while lesions on NahG plants often coalesced and had an average size of 150 mm^2. Interestingly, in NahG plants, the lesions were not confined to the inoculated leaf but would continue to expand, moving out of the leaf, through the petiole to the stem, where necrosis spread bidirectionally along the stem (Fig. 2). The expansion of the lesions was accompanied by the presence of TMV as determined by RNA blot analysis. However, the virus did not gain access to the phloem and spread systemically as would be observed in tobacco cultivars that do not carry N-gene resistance. Instead, the virus apparently spread in a cell-to-cell manner. The enhanced disease susceptibility of NahG tobacco was not specific to TMV but also extended to fungal (i.e., *C. nicotianae, Phytophthora parasitica*) and bacterial (i.e., *Pseudomonas syringae* pv. *tabaci*) pathogens.

To determine if the enhanced susceptibility extended to other plants, *Arabidopsis thaliana* ecotype "Columbia" (Col-O) was transformed with the same *nahG* construct as

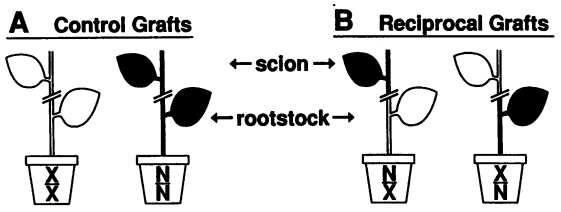

FIG. 1. Schematic representation of the grafting experiments with Xanthi-nc and NahG tobacco plants. The rootstocks of the grafted plants were either inoculated with TMV or mock-inoculated with buffer. After 7 days scions were challenge inoculated with TMV or *C. nicotianae*. (*A*) Control grafts in which Xanthi-nc or NahG scions are grafted onto rootstocks of Xanthi-nc or NahG, respectively. (*B*) Reciprocal grafts in which NahG scions are grafted onto Xanthi-nc rootstocks and Xanthi-nc scions are grafted onto NahG rootstocks. X, Xanthi-nc; N, NahG.

Table 2. Induction of SAR in grafted tobacco plants

Graft*	Inducer†	Average lesion size,‡ mm	Average %§	Average % infection¶
X/X	Buffer	1.49 ± 0.24		
X/X	TMV	0.63 ± 0.29	41	17
N/N	Buffer	2.23 ± 0.34		
N/N	TMV	2.22 ± 0.31	99	101
N/X	Buffer	2.01 ± 0.29		
N/X	TMV	2.11 ± 0.33	104	118
X/N	Buffer	1.58 ± 0.28		
X/N	TMV	0.63 ± 0.24	40	12

*Grafts are denoted as scion above the dividing line and rootstock below the line; X, Xanthi-nc; N, NahG.
†Seven days posttreatment of rootstock leaves with buffer or TMV, scion leaves were assayed for SAR by challenge inoculation with TMV or *C. nicotianae*.
‡TMV lesions on the challenge leaves were measured (average diameter ± SD) at 5–10 days after challenge; an average of four experiments is shown.
§The size of TMV lesions on the TMV-induced plants is expressed relative to the buffer-pretreated plants; an average of four independent experiments is shown.
¶The *C. nicotianae*-infected area on the TMV-induced plants is expressed relative to the buffer-treated plants; an average of two experiments is presented.

used in tobacco. These plants were tested for resistance to bacterial and fungal pathogens of *Arabidopsis*. NahG *Arabidopsis* showed enhanced disease susceptibility to both *Pseudomonas syringae* pv. tomato (*Pst*) and *Peronospora parasitica*. The NahG plants supported significantly more growth of both bacterial and fungal pathogens than wild-type plants and exhibited much more severe disease symptoms. These results were consistent with those from the NahG tobacco lines, indicating that plants transformed with NahG have enhanced susceptibility to viral, bacterial, and fungal pathogens.

nahG Expression Suppresses Genetic Resistance

Arabidopsis NahG plants express higher levels of both *nahG* RNA and protein than NahG tobacco plants. To better understand the role of SA and SAR in disease resistance, we determined whether genetic resistance was suppressed in these plants. Genetic disease resistance has been demonstrated for interactions between certain *Arabidopsis* ecotypes and particular bacterial and fungal species. For example, the *Arabidopsis* ecotype "Col-O" has been shown to be a host for the bacterial pathogen *Pseudomonas syringae* pv. tomato, strain DC3000. However, Col-O contains a gene that confers resistance against the bacterial avirulence gene, *avr*Rpt2. When *Arabidopsis* ecotype Col-O was inoculated with *Pst*DC3000, chlorotic spots symptomatic of bacterial speck disease develop over the course of 7 days; bacterial titer increased four to five orders of magnitude in the same period of time. When Col-O plants were inoculated with *Pst*DC3000 containing the *avr*Rpt2 gene, resistance lesions (HR) resulted and bacterial titer increased only 50- to 100-fold. In striking contrast, NahG plants inoculated with the same bacteria exhibited severe disease symptoms; these were accompanied by an increase in bacterial titer of four to five orders of magnitude. Thus, growth of avirulent bacteria in NahG plants was similar to growth of virulent bacteria on susceptible plants. Suppression of disease resistance in NahG was also seen with *Pseudomonas syringae* pv. *maculicola* ES4326 containing *avr*Rpt2, *avr*Rpm1, or *avr*B, suggesting that the enhanced susceptibility of NahG plants was not limited to particular pathovars or specific avirulence genes.

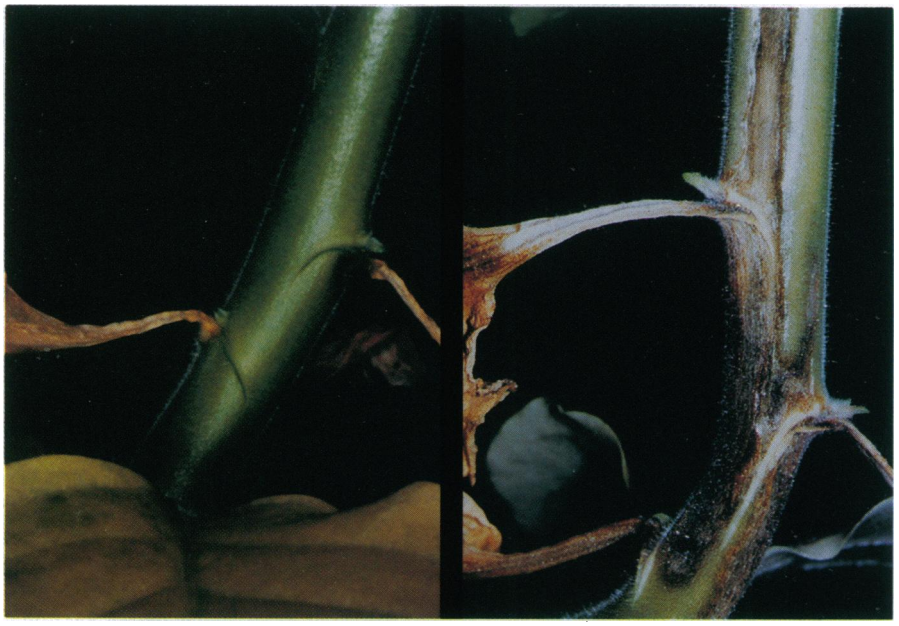

FIG. 2. Increase in TMV disease symptoms in NahG tobacco. In Xanthi-nc leaves the TMV lesions are limited to the infected leaves (*Left*) while the lesions in NahG plants spread out of the leaf to the stem (*Right*). Plants are shown at 19 days postinoculation with TMV.

In further experiments, we compared susceptibility of the NahG plants to Col-O wild-type plants following inoculation with various isolates of the fungal pathogen *Peronospora parasitica*. When Col-O were inoculated with the "Wela" isolate, which is virulent on the *Arabidopsis* ecotype "Landsberg" (Ler) but avirulent on Col-O, no growth of the fungus was observed. In contrast, Col-O NahG plants supported abundant growth of the "Wela" isolate. Histological examination of the infected leaves showed that the leaf was riddled with fungal hyphae and exhibited abundant conidiophores and oospores. Moreover, when Col-O or Ler was inoculated with a compatible isolate (i.e., "Noco" or "Wela," respectively), the plant eventually outgrew the infection. In contrast, NahG plants cannot outgrow the fungus and eventually succumb to the disease (Fig. 3). Inoculation of NahG with the fungal isolate "Emwa," which is incompatible with Col-O, also resulted in extensive fungal growth and eventual plant death. Therefore, we conclude that high levels of *nahG* gene expression suppresses the action of host resistance (R) genes against bacterial and fungal pathogens. At this point, we believe that this effect is due to the lack of SA accumulation in these plants.

Role of H_2O_2 in SAR Signal Transduction

Chen *et al.* (24, 25) have reported the isolation of a SA-binding protein (SABP). The purified protein was reported to have a K_d for SA of 14 μM (26). This K_d is consistent with the levels of SA measured in TMV-infected tissue. The cDNA encoding a related protein was isolated and shown by DNA sequence analysis to have strong sequence homology to catalase (26). The SABP was also shown to have catalase activity that was inhibited by SA, supporting the conclusion that the SABP was indeed a catalase isozyme (26). Because treatment of leaves with 3-aminotriazole (3-AT), paraquat, or glycolate, compounds that generate H_2O_2, could lead to PR-1 protein accumulation, the authors suggested that SA leads to H_2O_2 accumulation, which, in turn, induces SAR. However, there were several important issues raised by this model. First, H_2O_2

FIG. 3. Comparison of disease symptoms caused by *Peronospora parasitica* race "Wela" on resistant and susceptible ecotypes of *Arabidopsis*. (*Left*) Compatible ecotype Landsberg. (*Right*) Resistant ecotype Col-O. (*Center*) Col-O expressing *nahG*, showing lethal disease progression. Plants are shown at 21 days postinoculation.

was measured only in the SA-treated leaves, where there was a 40% increase; H_2O_2 in uninfected leaves of TMV-inoculated plants was not measured. Second, the H_2O_2 inducers used in the study were herbicides and SAR following herbicide treatment was not measured. The herbicidal action of both 3-AT and paraquat is closely linked to their ability to generate active oxygen species. Thus, chemical treatments leading to H_2O_2 accumulation would be expected to induce death in tissues where PR-1 was accumulating. Finally, while SA levels as high as 6 μg/g of fresh weight or 54 μM have been measured in cell layers adjacent to the lesion, in tissue 1 cm distal to the lesion, and in uninfected, systemic tissue expressing SAR and SAR genes, the levels were 10–100 times lower (27). Thus, only the SA concentration around the lesion was consistent with the K_d of the enzyme. This result was surprising since SAR gene expression in systemic tissue could reach 25–50% of the level observed around the lesion (5).

To determine if H_2O_2 was induced in systemic tissue at a time when SAR was expressed, we infected leaves with TMV and then measured H_2O_2 concentration, SAR gene expression, and SAR. We found that, although both SAR and SAR gene expression were detected consistently, the levels of H_2O_2 did not increase. Thus, we find no evidence to support the involvement of H_2O_2 in the induction of SAR.

To better understand the action of 3-AT, paraquat, and glycolate, we treated leaves with these chemicals as described by Chen *et al.* (26). After 7 days we measured both SAR gene induction and resistance to TMV. In Xanthi tobacco there was a small increase in PR-1a mRNA following treatment, but the significance of the increase was questionable since the H_2O control also slightly induced the PR-1a gene. However, there was no measurable increase in resistance in the treated leaves with the exception of leaves treated with paraquat. As controls in these experiments leaves were also treated with SA and 2,6-dichloroisonicotinic acid (INA), a potent inducer of SAR. Both SAR gene expression and resistance to TMV were high in the INA-treated leaves.

The same experiments were carried out in NahG tobacco plants. In this case, the levels of PR-1a mRNA and resistance were only induced by INA. Thus, it appears that the induction of PR-1a and resistance in these experiments is dependent on the accumulation of SA.

Based on these results we believe that the SA inhibition of catalase is important in the tissue surrounding a lesion. H_2O_2 may induce SA, which can then inhibit catalase, resulting in more H_2O_2 accumulation. This type of cycling would serve to enhance H_2O_2 accumulation and could thus potentiate damage by free radicals and ensuing cell death. However, the role of H_2O_2 in SAR and SAR gene induction remains questionable.

Summary

Plants have evolved many defenses that act together to promote the health of the organism. One component is the pathogen-inducible, systemic resistance called SAR. The signaling pathway for SAR, genetic resistance, and disease susceptibility are all affected by accumulations of SA. The importance of the SA-dependent pathways in plant health is demonstrated in plants engineered to express salicylate hydroxylase. Plants that can no longer accumulate SA are compromised in their ability to withstand pathogen infection. Thus, SA-dependent signal transduction plays a central role in plant defense against pathogens and will undoubtedly serve as a paradigm for defense signaling in plants.

1. Ryals, J., Uknes, S. & Ward, E. (1994) *Plant Physiol.* **104,** 1109–1112.
2. Kuc, J. (1982) *BioScience* **32,** 854–860.
3. Chester, K. S. (1933) *Q. Rev. Biol.* **8,** 275–324.
4. Ross, A. F. (1961) *Virology* **14,** 340–358.
5. Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J.-P. & Ryals, J. A. (1991) *Plant Cell* **3,** 1085–1094.
6. Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H. & Ryals, J. (1995) *Mol. Plant Microbe Interact.*, in press.
7. Bol, J. F., Linthorst, H. J. M. & Cornelissen, B. J. C. (1990) *Annu. Rev. Phytopathol.* **28,** 113–138.
8. Bowles, D. J. (1990) *Annu. Rev. Biochem.* **59,** 873–907.
9. Carr, J. P. & Klessig, D. F. (1989) in *Genetic Engineering*, eds. J. K. Setlow (Plenum, New York), Vol. 11, pp. 65–109.
10. Linthorst, H. J. M. (1991) *Crit. Rev. Plant Sci.* **10,** 123–150.
11. Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl Goy, P., Luntz, T., Ward, E. & Ryals, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7327–7331.
12. Malamy, J., Carr, J. P., Klessig, D. F. & Raskin, I. (1990) *Science* **250,** 1002–1004.
13. Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. & Inverardi, B. (1990) *Science* **250,** 1004–1006.
14. Yalpani, N., Shulaev, V. & Raskin, I. (1993) *Phytopathology* **83,** 702–708.
15. White-Stevens, R. H. & Kamin, H. (1972) *J. Biol. Chem.* **247,** 2358–2370.
16. Yen, K.-M. & Gunsalus, I. C. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 874–878.
17. You, I.-S., Ghosal, D. & Gunsalus, I. C. (1991) *Biochemistry* **30,** 1635–1641.
18. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227,** 1229–1231.
19. Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. & Ryals, J. (1994) *Plant Cell* **6,** 959–965.
20. Jenns, A. E. & Kuc, J. (1979) *Phytopathology* **7,** 753–756.
21. Tuzun, S. & Kuc, J. (1985) *Physiol. Plant Pathol.* **26,** 321–330.
22. Delaney, T., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. & Ryals, J. (1994) *Science* **266,** 1247–1250.
23. Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. & Ryals, J. (1993) *Science* **261,** 754–756.
24. Chen, Z. & Klessig, D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8179–8183.
25. Chen, Z., Ricigliano, J. W. & Klessig, D. F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9533–9537.
26. Chen, Z., Silva, H. & Klessig, D. F. (1993) *Science* **262,** 1883–1885.
27. Enyedi, A. J., Yalpani, N., Silverman, P. & Raskin, I. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2480–2484.