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Induction, modification, and transduction of the salicylic acid signal in plant defense responses

(active oxygen species/catalase/disease resistance/pathogenesis-related proteins/signal transduction)

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ABSTRACT Studies in our laboratory as well as others strongly suggest that salicylic acid (SA) plays an important signaling role in plant defense against pathogens. We have found that increases in endogenous SA levels correlates with both resistance of tobacco to infection with tobacco mosaic virus and induction of defense-related genes such as that encoding pathogenesis-related protein 1 (PR-1). Some of this newly synthesized SA was conjugated to glucose to form SA β -glucoside. A cell wall-associated β -glucosidase activity that releases SA from this glucoside has been identified, suggesting that SA β -glucoside serves as an inactive storage form of SA. By purifying a soluble SA-binding protein and isolating its encoding cDNA from tobacco, we have been able to further characterize the mechanism of SA signaling. This protein is a catalase, and binding of SA and its biologically active analogues inhibited catalase's ability to convert H_2O_2 to O_2 and H_2O . The resulting elevated levels of cellular H_2O_2 appeared to induce PR-1 gene expression, perhaps by acting as a second messenger. Additionally, transgenic tobacco expressing an antisense copy of the catalase gene and exhibiting depressed levels of catalase also showed constitutive expression of PR-1 genes. To further dissect the SA signaling pathway, we have tested several abiotic inducers of PR gene expression and disease resistance for their ability to stimulate SA production. Levels of SA and its glucoside rose following application of all of the inducers except 2,6-dichloroisonicotinic acid. 2,6-Dichloroisonicotinic acid was found to bind catalase directly and inhibit its enzymatic activity. Thus, it appears that many compounds that induce PR gene expression and disease resistance in plants inactivate catalases directly or indirectly.

Infection of tobacco (*Nicotiana tabacum*) with tobacco mosaic virus (TMV) is one of the best characterized systems for analysis of plant-pathogen interactions. Following infection the host plant develops one of two distinct responses, depending both on its genetic background and on that of the viral strain. In a susceptible tobacco cultivar (e.g., Xanthi), infection with TMV (e.g., strain U1) leads to the systemic spread of virus from the site of entry to distal parts of the host, with the potential for causing widespread damage. By contrast, in tobacco cultivars (e.g., Xanthi nc) that carry a dominant resistance gene (e.g., *N* locus), the spread of TMV is restricted to a small zone of tissue around the site of entry, where a necrotic lesion later appears. This resistance phenotype is called the hypersensitive response (HR) and is frequently accompanied by the subsequent induction throughout the

plant of systemic acquired resistance (SAR). SAR provides protection throughout the plant against a broad spectrum of pathogens for up to several months.

Correlated with both the HR and SAR is the synthesis of abundant amounts of host-encoded, pathogenesis-related (PR) proteins. The production of these proteins seems to be part of a general defense system against pathogens, for their synthesis can also be induced by certain bacteria and fungi. In addition to serving as markers for disease resistance, several of the tobacco PR proteins have been shown to have antimicrobial activity (for review see ref. 1).

During the past five years a large body of evidence has been obtained which suggests that salicylic acid (SA) plays an important role in the induction of plant disease resistance. Here we briefly review our early studies on the induction and modification of the SA signal in TMV-infected tobacco. The identification and characterization of a SA receptor and definition of one of SA's modes of action are discussed in more detail.

Induction of the SA Signal

Treatment of plants with exogenous SA has been shown to affect a variety of processes. Most germane to our studies was the observation first made by White (2) that SA or acetylsalicylic acid induced PR protein synthesis and enhanced resistance to a number of pathogens, including TMV (for review see refs. 3 and 4). This led Van Loon (5) to postulate that SA acted by mimicking an endogenous phenolic signal. However, we speculated that SA itself was the natural signaling compound for PR gene expression during the resistance responses of tobacco to TMV infection. To test this we monitored the endogenous levels of SA following TMV infection (6) and found that SA levels rose specifically during the resistance responses (HR and SAR). Moreover, induction of PR-1 (and PR-2) gene expression paralleled the rise in SA levels in both

Abbreviations: INA, 2,6-dichloroisonicotinic acid; HR, hypersensitive response; PR, pathogenesis-related; SA, salicylic acid; SAG, SA β -glucoside; SABP, SA-binding protein; SAR, systemic acquired resistance; TMV, tobacco mosaic virus.

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inoculated and uninoculated leaves of infected resistant plants. In susceptible plants, neither an increase in endogenous SA nor induction of PR gene expression was observed. These results suggested that SA might play a role in the development of systemic as well as local resistance.

The ability of TMV-resistant tobacco to synthesize SA was also shown to correlate with resistance. At elevated temperatures (>28°C), TMV-infected plants do not synthesize PR proteins or undergo a HR but become systemically infected. We demonstrated that at these high temperatures SA levels did not rise after infection (7), consistent with the model that SA is part of the signal transduction pathway leading to disease resistance. When these plants were subsequently shifted to 22°C, which restores their ability to express the PR genes and develop a HR, SA production increased rapidly and dramatically. This increase preceded both PR gene induction and the HR.

Evidence That SA Plays a Role in Defense

Additional evidence that SA plays an important role in disease resistance in tobacco has been provided by Ryals and coworkers (8), who found that the same set of defense-related genes expressed both at the site of TMV infection and subsequently throughout the plant was also activated by SA treatment. Moreover, transgenic tobacco plants which expressed the *nahG* gene, encoding the SA-metabolizing enzyme salicylate hydroxylase from *Pseudomonas putida*, accumulated little or no SA after TMV infection and were defective in their ability to establish SAR (9). The major evidence for a role of SA in disease resistance in tobacco may be summarized as follows. (i) Application of exogenous SA induces expression of defense-related genes (e.g., PR genes) and partial protection against pathogens. (ii) Increases in endogenous SA correlate with expression of defense-related genes and development of resistance. (iii) SA induces the same set of nine genes that are activated systemically after TMV infection. (iv) Expression of the salicylate hydroxylase gene in transgenic tobacco plants blocks accumulation of SA and prevents development of SAR.

Modification of the SA Signal

We (7) and Raskin's group (10) found that some of the SA synthesized following TMV infection was subsequently converted to a conjugated form. The majority of these conjugates appeared to be SA β -glucoside (SAG), based on their hydrolysis by β -glucosidase. SAG was present in only very low levels in uninfected tobacco. However, infection led to high levels of SAG due to conjugation of newly synthesized SA to glucose. SAG was also formed when plants were treated with exogenous SA.

The existence of SAG suggests additional complexity in the modulation of the SA signal, since many biologically active compounds in plants (e.g., auxin, cytokinins, and gibberellins) are inactivated via conjugation with glucose. To determine whether SAG is also inactive and whether it plays a role in plant defense, we chemically synthesized SAG (11). Following injection of synthetic SAG into tobacco leaves, PR-1 gene expression was observed. However, since subsequent experiments indicated that SAG was rapidly hydrolyzed in the extracellular space of injected leaves, we suspect that the released free SA was the active signal for induction of PR genes. A β -glucosidase activity that is associated with the cell wall and can be released with high salt has since been identified. As shown in Table 1 it has broad substrate specificity. Consistent with the idea that SAG is inactive is the observation that tobacco suspension cells are able to take up SA, but not SAG. From the above data we postulate that SAG plays a role in SAR, serving as an inactive storage form which

Table 1. Substrate specificity of β -glucosidases

Substrate	β -Glucosidase activity, %	
	Tobacco	Almond
1. SAG	100	100
2. Indoxyl β -D-glucoside	36	95
3. 4-Methylumbelliferyl β -D-glucoside	163	94
4. Hydroxychalcone β -D-glucopyranoside (Arbutin)	41	97
5. Phenyl β -D-glucopyranoside	25	116
6. Methyl β -D-glucopyranoside	0	30
7. D-(+)-Cellobiose	344	83
8. <i>p</i> -Nitrophenyl β -D-glucopyranoside	155	103
9. <i>p</i> -Nitrophenyl α -L-arabinopyranoside	13	72
10. <i>p</i> -Nitrophenyl α -L-arabinofuranoside	69	27
11. <i>p</i> -Nitrophenyl β -D-galactopyranoside	97	67

Crude cell wall preparations were obtained from 7-week-old tobacco (*N. tabacum* cv. Xanthi nc) plants as follows. Freshly harvested leaf tissue (3.6 g) was frozen in liquid nitrogen and immediately homogenized with mortar and pestle in 8 ml of 50 mM acetate buffer (pH 4.5). After centrifugation at 48,000 $\times$ g for 20 min, the pellet was washed with 8 ml of deionized water and resuspended in 4 ml of 50 mM acetate buffer for β -glucosidase activity assays. One hundred microliters of crude cell wall preparation or 25 μ g of a commercial almond β -glucosidase (Sigma) dissolved in 50 mM acetate buffer was mixed in a total volume of 500 μ l 50 mM acetate with 2 mM (final concentration) substrate and incubated at 37°C with agitation (100 rpm) for 4 hr. The enzyme reaction was stopped by addition of Na₂CO₃ to 1 M. Enzyme activities with substrates 1–8 were determined by measuring the concentration of glucose in the supernatant with a commercial glucose detection kit (Sigma). Enzyme activities with substrates 8–11 were determined by comparing the absorbances at 405 nm of the supernatants with a concentration curve for *p*-nitrophenol. The activities were normalized to that found with SAG as the substrate.

can be rapidly cleaved to release active SA at the site of a challenging infection.

Perception of the SA Signal by Catalase

To elucidate SA's mechanism of action in plant signal transduction, we have identified and recently purified and characterized a salicylic acid-binding protein (SABP) from tobacco leaves (12, 13). The SABP is a 240- to 280-kDa complex which contains a 57-kDa subunit. There are two characteristics which suggest that SABP is the SA receptor. The first is its affinity. It binds SA with an affinity ($K_d = 14 \mu$ M) consistent with the range of physiological concentrations (1–50 μ M) of SA observed during the induction of defense responses. The second is its binding specificity. SABP can bind only SA and its analogues which are able to induce PR genes and resistance. It does not bind biologically inactive analogues.

A cDNA clone encoding the 57-kDa subunit was isolated by screening a λ gt11 expression library with monoclonal antibodies directed against SABP (14). Surprisingly, the amino acid sequence encoded by this clone revealed high homology to catalases. Several other lines of evidence also indicate that SABP is a catalase. The sequence of five tryptic peptides isolated from SABP matched those of catalases. Also, the size of the SABP complex and its 57-kDa subunit are consistent with the known structure of catalases, which are tetrameric enzymes composed of four identical or similar subunits of 55–60 kDa. Finally, purified SABP was highly active for the conversion of H₂O₂ to O₂ and H₂O, which is the function of catalases.

SA was found to block the catalase activity of purified SABP; it also inhibited catalase activity in crude tobacco leaf extracts and of tobacco suspension cells. Furthermore, the effectiveness of different SA analogues to inhibit the catalase activity of SABP correlated with their abilities to bind to SABP

and to induce defense responses. These results suggest that binding of SA by SABP is responsible for inhibition of its catalase activity and induction of defense responses.

In plants, H_2O_2 production is an ongoing process, being synthesized as a by-product of photorespiration, β -oxidation of fatty acids, photosynthesis, and oxidative phosphorylation (Fig. 1). This suggested the possibility that SA signals the activation of defense responses by altering cellular H_2O_2 levels. In support of this hypothesis, we observed that SA treatment of tobacco leaves resulted in elevated H_2O_2 levels, as did treatment with an inhibitor of plant and animal catalases, 3-amino-1,2,4-triazole. In contrast, treatment with the SA analogue 3-hydroxybenzoic acid failed to induce either increases in H_2O_2 levels or PR-1 gene expression, consistent with its inability to bind to SABP or inhibit SABP's catalase activity.

When H_2O_2 levels were elevated by injecting leaves with H_2O_2 or 3-amino-1,2,4-triazole, expression of PR-1 genes was activated. PR-1 gene expression was also induced by injection of compounds that promote H_2O_2 synthesis *in vivo*, such as paraquat. Together, the above results argue that SA acts by blocking catalase activity, which leads to elevated H_2O_2 levels. H_2O_2 , or another active oxygen species derived from it, then activates defense-related genes such as PR-1 genes, perhaps by acting as a second messenger (Fig. 1).

Catalase Antisense Transgenic Tobacco

To provide additional support for our model that SA activates plant defenses via inhibition of catalase activity, we have constructed transgenic tobacco plants that constitutively express an antisense copy of the cloned catalase gene (Z.C. and D.F.K., unpublished results). If SA activates the plant's defense responses by inhibiting catalase activity, then inhibiting catalase synthesis with antisense RNA should mimic this effect. Preliminary results indicate that the catalase antisense transgenic lines which showed significant reduction in catalase protein levels constitutively expressed the PR-1 genes. Thus, blocking catalase action either by inhibiting its activity via SA or inhibiting its synthesis via genetic engineering leads to induction of defense responses.

SA action in plant defense is not restricted to incompatible TMV-tobacco interactions (for review see ref. 4). To provide

support for the basic and general character of SA's proposed mechanism of action in tobacco, we have demonstrated that other plant species, including cucumber, tomato, and *Arabidopsis*, also contain a SA-binding activity and SA-inhibitable catalase activity (15).

Mechanisms of Action of Abiotic Inducers

We have found that several abiotic inducers of PR gene expression and enhanced resistance (polyacrylic acid, thiamine, α -aminobutyric acid, and $BaCl_2$) appear to act through SA, since treatment of tobacco with these compounds stimulated production of SA and its glucoside. A noted exception is INA (J.M., P.S.-C., J.H., A. Guo, and D.F.K., unpublished data). A hint as to INA's mechanism of action came from the work of Kirkman and Gaetani (16), who demonstrated that mammalian catalases tightly bind NADPH and NADH. These cofactors and INA share the nicotinic acid moiety, suggesting that INA might also bind catalase. However, we suspected that INA binding—in contrast to the binding of NADPH/NADH, which does not inhibit the enzyme's activity (16)—would block catalase activity just as observed for SA.

Indeed, we observed that INA bound catalase and inhibited its activity (17). Moreover, as for SA, there are biologically active and inactive analogues of INA for the induction of PR gene expression and enhanced disease resistance. Only the biologically active analogues effectively bound to catalase and inhibited its enzymatic activity. Thus, many compounds that induce PR gene expression and disease resistance, directly or indirectly, appear to target catalase.

Conclusion

SA appears to be a very important signal for the activation of certain plant defense responses. Our results strongly argue that one mode of action of SA is its inhibition of catalase activity, although SA may also have additional mechanisms of action that are yet to be discovered. In addition, we believe that catalase can be considered a receptor for SA. It perceives the SA signal by binding this ligand and then transduces this signal by inhibiting its own enzymatic activity, which leads to elevated H_2O_2 levels.

FIG. 1. Metabolism of H_2O_2 in plants. Two major sources of H_2O_2 are photorespiration and β -oxidation of fatty acids. H_2O_2 is also produced from superoxide anions (O_2^-) by superoxide dismutase (SOD). The superoxide anion is formed by transfer of electrons (e^-) to molecular oxygen. These misdirected electrons often originate from electron transport during oxidative phosphorylation in the mitochondria and during photosynthesis in the chloroplasts. Membrane-bound, NADPH-dependent oxidases also generate H_2O_2 . This is probably an important local source of H_2O_2 at the site of injury or infection. H_2O_2 can be converted to other active oxygen species (AOS) such as the highly reactive hydroxyl radical. H_2O_2 and other AOS derived from it may be involved in a number of defense responses, including the induction of defense-related genes such as the PR-1 genes. It may also lead to other responses, yet to be defined. There are two major pathways involved in the breakdown of H_2O_2 . H_2O_2 can be converted to H_2O and O_2 by catalase, a process that can be inhibited by SA or 2,6-dichloroisonicotinic acid (INA). The other pathway involves breakdown of H_2O_2 by specific peroxidases either in the course of normal cell metabolism (e.g., in lignin formation) or via a specific pathway to eliminate toxic levels of H_2O_2 (e.g., Halliwell-Asada pathway). [Reproduced from ref. 4 with permission, copyright 1984, Kluwer Academic Publishers.]

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