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Colloquium Paper

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Biosynthesis and metabolism of salicylic acid

(systemic acquired resistance/salicylic acid glucoside/benzoic acid 2-hydroxylase/UDP-glucose:salicylic acid glucosyltransferase)

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ABSTRACT **Pathways of salicylic acid (SA) biosynthesis and metabolism in tobacco have been recently identified. SA, an endogenous regulator of disease resistance, is a product of phenylpropanoid metabolism formed via decarboxylation of *trans*-cinnamic acid to benzoic acid and its subsequent 2-hydroxylation to SA. In tobacco mosaic virus-inoculated tobacco leaves, newly synthesized SA is rapidly metabolized to SA *O*-β-D-glucoside and methyl salicylate. Two key enzymes involved in SA biosynthesis and metabolism: benzoic acid 2-hydroxylase, which converts benzoic acid to SA, and UDPglucose:SA glucosyltransferase (EC 2.4.1.35), which catalyzes conversion of SA to SA glucoside have been partially purified and characterized. Progress in enzymology and molecular biology of SA biosynthesis and metabolism will provide a better understanding of signal transduction pathway involved in plant disease resistance.**

Several physiological and biochemical effects of salicylic acid (SA) applied to plants have been known for a long time. These include flowering induction (1), inhibition of phosphate and potassium uptake (2, 3), and inhibition of ethylene biosynthesis (4). However, the regulatory role of endogenous SA has only been documented for thermogenesis in *Arum* lilies (5) and for pathogen resistance in tobacco and cucumber (6, 7).

The interaction between plants and necrotizing pathogens often leads to the development of resistance to subsequent infection. This defense response is not only restricted to plant tissues in contact with the pathogen. Pathogen-free parts of the inoculated plant also become resistant. This phenomenon called systemic acquired resistance (SAR) was reviewed by Ryals *et al.* (8).

The development of SAR is usually preceded by a hypersensitive response characterized by the formation of necrotic lesions around the site of infection (for reviews, see refs. 9 and 10). This primary defense response in the inoculated parts of the plant is accompanied by an array of biochemical changes. These include generation of active oxygen species, cell death, overproduction of phenolics, deposition of lignin-related materials, and induction of the expression of pathogenesis-related (PR) proteins.

The occurrence of SAR in response to a pathogen requires a long-distance transport of a factor originating in the tissue expressing the hypersensitive response that moves systemically to other parts of the plant. It was suggested that SA is likely to be the molecule responsible for SAR of plants to pathogens (for reviews, see refs. 11 and 12).

The importance of SA as a component of a signal transduction pathway in disease resistance and as a regulator of thermogenesis has stimulated interest in its biosynthesis and metabolism. In this review, we discuss the biosynthetic and metabolic pathways of SA and the key enzymes involved in its biosynthesis and catabolism. Emphasis is placed on the biochemical events responsible for the accumulation and metabolism of SA during tobacco mosaic virus (TMV) infection of hypersensitively responding tobacco.

Biosynthesis of SA

Feeding experiments performed in the early 1960s suggested that in plants SA is synthesized from cinnamic acid by two possible pathways (13–15). One involves side-chain decarboxylation of cinnamic acid to benzoic acid followed by 2-hydroxylation to SA. Alternatively, cinnamic acid could be first 2-hydroxylated to *o*-coumaric acid and then decarboxylated to SA. It was suggested (16) that tomato seedlings infected with *Agrobacterium tumefaciens* synthesized SA via *o*-coumaric acid whereas the benzoic acid pathway operated in noninfected plants. In agreement with the *o*-coumaric acid pathway, leaves of *Gaultheria procumbens* or *Primula acaulis* accumulated both labeled *o*-coumaric acid and SA after feeding with ^{14}C-labeled cinnamic acid or phenylalanine (13, 14). However, leaves of both plants and potato tubers also converted carboxyl-labeled benzoic acid to SA (13, 15, 17). Recently, Yalpani *et al.* (18) conclusively demonstrated the biosynthetic pathway of SA in tobacco plants. ^{14}C-tracer studies with cell suspensions and mock- or TMV-inoculated tobacco leaves indicated that the label moves from cinnamic acid to SA via benzoic acid (Fig. 1). ^{14}C-labeled *o*-coumaric acid was not detected after feeding with labeled cinnamic acid. In addition, *o*-coumaric acid did not serve as a precursor of SA in tobacco (18). More recent data show that the cinnamic acid → benzoic acid → SA pathway also functions in rice seedlings (62). SA levels in healthy rice leaves are at least an order of magnitude higher than in tobacco (19).

Little is known about the mechanism by which cinnamic acids are decarboxylated to the corresponding benzoic acids. Two mechanisms have been proposed for this side-chain shortening reaction. The first mechanism may operate via β-oxidation similar to that observed in fatty acid catabolism. This hypothesis was supported by studies of cell-free extracts of *Quercus pedunculata* where the conversion of cinnamic acid to benzoic acid was stimulated by the addition of ATP and CoA (20). However, there is also evidence supporting a nonoxidative chain-shortening mechanism from studies of cell suspension cultures of *Vanilla planifolia* (21), *Lithospermum erythrorizum* (22), and *Daucus carota* (23).

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Abbreviations: SA, salicylic acid; SAR, systemic acquired resistance; BA2H, benzoic acid 2-hydroxylase; PR, pathogenesis related; SA GTase, UDPglucose:SA glucosyltransferase; TMV, tobacco mosaic virus.

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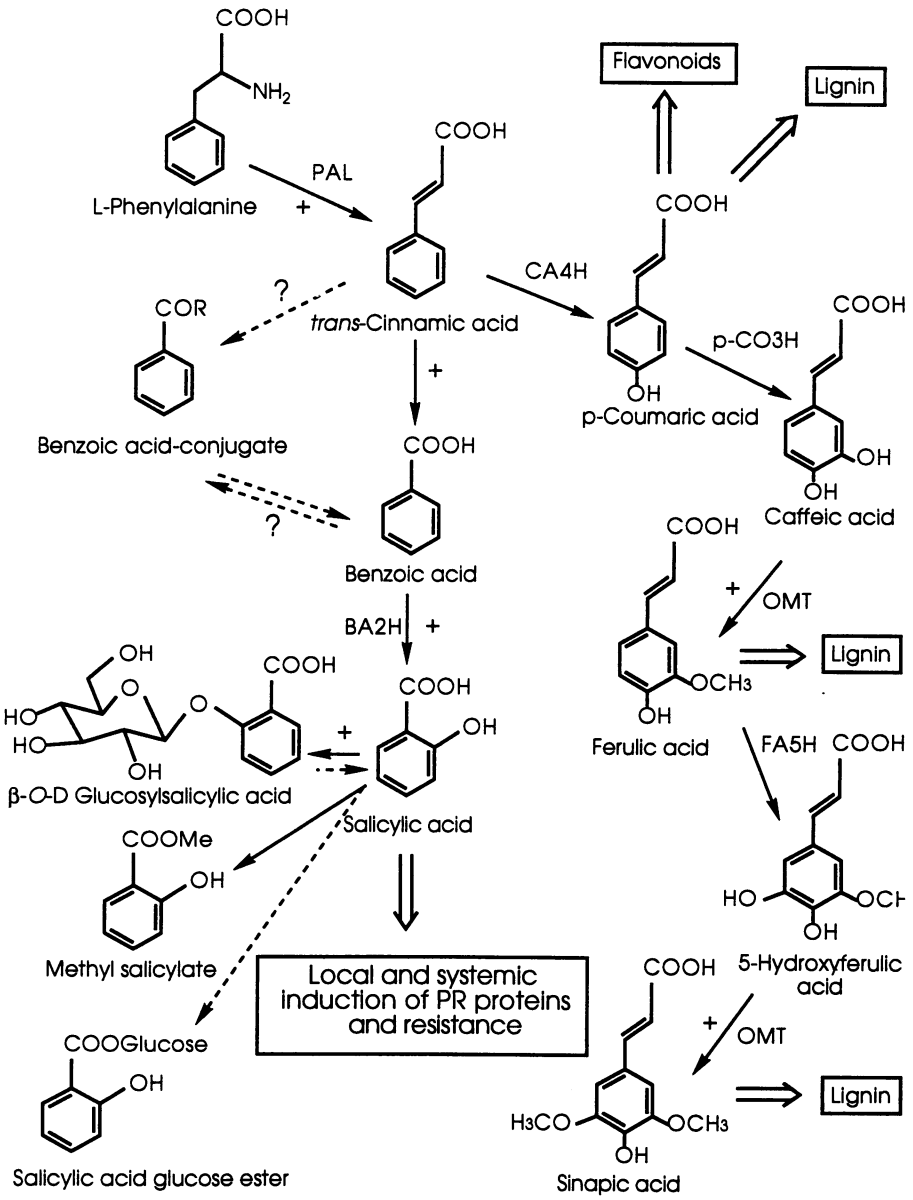

FIG. 1. Schematic diagram of biosynthetic and metabolic pathway of SA and phenylpropanoids.

Studies of *Melilotus alba* chloroplasts (24) and *Petunia hybrida* (25) provided the first clues about the mechanism by which cinnamic acid is 2-hydroxylated to form *o*-coumaric acid. León *et al.* (26) reported the identification of a benzoic acid 2-hydroxylase (BA2H) in cell-free tobacco leaf extracts. This enzyme specifically catalyzed the synthesis of SA from benzoic acid. BA2H activity was 10- or 6-fold induced after inoculation of tobacco plants with TMV or after infiltration of healthy plants with benzoic acid, respectively. The induction was a result of a *de novo* protein synthesis. In TMV-inoculated tobacco leaves, benzoic acid accumulation (18) paralleled the induction of BA2H activity (26). In addition, benzoic acid application induced BA2H activity in healthy tobacco plants (26). Therefore, it was proposed that an increase in the benzoic acid pool in inoculated tobacco leaves acts as the primary signal for the induction of BA2H and SA accumulation (27). BA2H is a monooxygenase that may belong to the cytochrome P450 superfamily. Unique properties of this enzyme include solubility and high molecular mass, ≈160 kDa (J.L., unpublished data).

Recently, an SA-binding protein from tobacco was purified (28) and it was suggested that this protein is involved in the signal transduction pathway of the plant disease resistance response. Chen *et al.* (29) isolated a cDNA encoding the SA binding protein, which was highly homologous to the genes encoding catalases. SA inhibited the catalase activity of the SA binding protein. Therefore, it was proposed that the accumulation of reactive oxygen species, brought about by the SA-induced inhibition of catalase, activates the expression of SAR genes such as those encoding PR proteins. However, the timing of hydrogen peroxide production and its reported activity may warrant another interpretation. It is known that the oxidative burst and peroxide production are very early events in pathogenesis that occur long before the accumulation of SA (30, 31). In addition, the concentration of SA in systemically protected tissues is significantly below the published K_d value of the SA binding protein (28). It is possible that the production of active oxygen is a cause and not the result of SA accumulation. Consistent with this view, UV and ozone treatments, which lead to the production of active oxygen species in plant tissues, increased BA2H activity, SA levels, and PR protein content of tobacco leaves (32). Moreover, hydrogen peroxide activated the BA2H activity *in vivo*, in infiltrated tobacco leaves, and *in vitro* in cell-free extracts from tobacco leaves. The rapid

activation of the BA2H activity resulted in a significant increase in SA levels in hydrogen peroxide-treated tobacco leaves (J.L. *et al.*, unpublished data).

Metabolism of SA

SA Glucoside and Glucose Esters. Many different types of hydroxybenzoic acid conjugates have been found in a wide range of plant species (33). It is not surprising that SA also forms conjugated products, mainly by glucosylation and less frequently by esterification (34). In an early study, trace amounts of SA and large amounts of SA glucosides were detected in *Helianthus annuus* hypocotyls fed with ^{14}C-labeled benzoic acid (15). Later, SA 2-*O*-β-D-glucoside (Fig. 1) was identified as a major SA metabolite in cell cultures of *Mallotus japonicus* (35, 36), extracts of spice plants belonging to the Apiaceae and Lamiaceae (37–39), and oat (40, 41) and bean (42) roots. SA endogenously synthesized as a result of TMV inoculation of tobacco leaves is also rapidly converted to SA glucoside, which accumulates in and around hypersensitive lesions (43, 44). Although SA 2-*O*-β-D-glucoside is the predominant SA conjugate in plants, other metabolites could be formed by esterification or additional hydroxylation of the aromatic ring. In soybean cell cultures fed ^{14}C-labeled SA or benzoic acid, glucose esters of SA were formed (45). Meanwhile, 2,5-dihydroxybenzoic acid (gentisic acid) and 2,3-dihydroxybenzoic acid (*O*-pyrocatechuic acid) were detected in leaves of various plants fed radioactive SA (46) and in *Astilbe sinensis* and tomato plants incubated with [^{14}C]cinnamic and benzoic acids (16, 47). However, these experiments did not determine whether dihydroxybenzoic acid can also form glucosides, since samples were acid-hydrolyzed before analysis. The occurrence of dihydroxybenzoic acid glucosides was demonstrated in roots of buckwheat (*Fagopyrum esculentum*) where SA was 5-hydroxylated to 2,5-dihydroxybenzoic acid (gentisic acid), which was further glucosylated to gentisic acid 5-*O*-β-D-glucoside (42).

As mentioned above, it is now established that in tobacco both endogenously produced and exogeneously supplied SA are metabolized to the same conjugate, SA 2-β-D-glucoside. In healthy tobacco leaves, little if any SA glucoside is present (43). However, TMV-inoculated tobacco leaves accumulate significant amounts of β-glucosidase-hydrolyzable SA glucosides (up to 80% of total SA) in the areas surrounding necrotic lesions. Only small amounts of SA glucoside were detected in phloem exudates and uninoculated leaves of TMV-inoculated tobacco (43). Recently another detailed analysis of SA metabolism in tobacco was performed by feeding [7-^{14}C]SA to tobacco leaf disks (48). As expected, SA glucoside was identified as a major product. SA glucose ester (Fig. 1) was identified as a relatively minor slow-forming metabolite (48). Release of $^{14}CO_2$ coincident with accumulation of SA glucoside suggests that glucosylation of SA may precede decarboxylation and further metabolism to more simple phenolic compounds and, possibly, the breakdown of the aromatic ring of SA.

Conversion of SA to 2-*O*-β-SA glucoside is catalyzed by UDPglucose:SA glucosyltransferase (GTase), which requires UDPglucose as a glucose donor. GTase activity was detected in cell cultures of *Mallotus japonicus* (35), oat roots (40, 41), and tobacco leaves (49) after SA application. For TMV-inoculated tobacco, increase in SA GTase activity coincided with the accumulation of SA and the formation of its product, SA glucoside. The highest enzymatic activity was detected in the vicinity of hypersensitive lesions where SA glucoside accumulated. Thus, the spatial and temporal distribution of SA GTase correlated with that of SA glucoside. SA GTase was partially purified and characterized in oat roots and tobacco leaves (40, 49). The molecular mass of tobacco SA GTase is close to 48 kDa (H.L. and I.R., unpublished data) while the oat enzyme is 50 kDa (40). These numbers are in agreement with molecular masses of other known GTases that range from 40 to 60 kDa. In oat and tobacco, SA GTase is highly specific to SA both as substrate and inducer. Previously identified GTases are localized in the cytoplasm or are associated with cell membrane. Tobacco and oat SA GTase activity is detected in the soluble protein fraction, suggesting that SA GTase is a cytoplasmic enzyme. SA induction of tobacco and oat SA GTases is inhibited by cycloheximide, a protein synthesis inhibitor (41, 49), and by RNA synthesis inhibitors in cell suspension cultures of *Mallotus japonicus* and in oat roots (35, 41). These results imply that the induction of SA GTase by SA is regulated at the transcriptional level.

The *as-1* element, located between positions −83 and −63 of the cauliflower mosaic virus 35S promoter was shown to be highly responsive to SA. A substantial induction of β-glucuronidase (GUS) mRNA was obtained by treating transgenic tobacco containing an AS-1–GUS fusion with SA. The induction was rapid and insensitive to cycloheximide in contrast to the late induction of PR genes by SA (50). So far no early SA-responsive genes have been identified in plants. The SA GTase gene could belong to this early response group, since SA GTase activity is induced before PR proteins in SA-infiltrated or TMV-inoculated tobacco plants.

Methyl Salicylate. Methyl salicylate (Fig. 1), a volatile SA ester, is found in oil of wintergreen (51), in the leaves of oats (52), red clover (53), fig (54), and tobacco (55) and in the volatile fractions of such fruits as plum, strawberry, black cherry, and tomato (56). Little is known about the biosynthesis of methyl salicylate. Recently however, it was discovered that large amounts of volatile methyl salicylate are released from TMV-inoculated tobacco in parallel with tissue accumulation of SA (V. Shulaev, personal communication). Almost no methyl salicylate evolved from healthy or mechanically wounded tobacco leaves. The quantities of methyl salicylate synthesized in TMV-inoculated tobacco indicate that this compound may be a major metabolite of SA.

Amino Acid SA Conjugate. It is well established that plant hormones form various metabolic products including amino acid conjugates, e.g., indole-3-acetyl-L-aspartate (57). *N*-Salicyloyl aspartic acid was identified in wild grapes (*Vitis riparia* and *Vitis rupestris*), some of their cross-bred hybrids (58), and French beans (59). It is not clear whether this compound is a metabolic product of SA or whether it is formed via other intermediates.

Role of SA Conjugates. At least in tobacco, SA is phytotoxic in concentrations >0.1 mM (I.R., unpublished data). However, conjugation may play a role in the detoxification of SA. SA glucoside appears to be inactive as an inducer of PR proteins (43, 60). It is also not required for the induction of resistance and PR proteins in tobacco, since it is mainly present in and around hypersensitive lesions and is rarely detected in systemically protected tissues. In addition, the absence of SA glucoside in phloem exudates makes it an unlikely candidate for the translocatable form of SA.

In addition to lowering cellular SA, SA glucoside may function as a slow-release storage form of SA that maintains SAR over extended periods of time. Similar storage mechanisms are known for other plant hormones. For example, cytokinin β-*O*-glucoside, a hypothesized storage compound, is hydrolyzed by a specific β-glucosidase to release active cytokinin (61). Another possibility is that the formation of SA glucoside is an irreversible reaction and a first step in SA catabolism.

It is tempting to speculate that volatile methyl salicylate, another major metabolite of SA in tobacco, may function as an airborne signal for both intra- and interplant communication. Treatment of tobacco plants with gaseous methyl salicylate resulted in an increase in tissue SA and PR proteins. In addition, tobacco plants gassed with methyl salicylate had

greater resistance to TMV (P. Silverman, personal communication).

Future Directions. SA is one of the mediators in a signal transduction pathway of disease resistance. However, it has not been determined whether SA or another signal(s) is the primary long-distance signal molecule in SAR. Moreover, little is known about the biochemical events that link pathogen-induced necrosis to SA accumulation. In particular, a connection should be established between the oxidative burst that follows pathogen recognition and the accumulation of SA. There is limited information about regulation and rate limiting steps of SA biosynthesis and metabolism. It is also unknown whether SA biosynthesis and metabolism vary in different plant species. SA levels in plants can be increased by enhancing the expression of genes encoding SA biosynthesis or by blocking the expression of genes involved in SA metabolism. Therefore, there is a need to identify and characterize SA biosynthetic and metabolic enzymes and to isolate their genes. In addition, it is important to identify subcellular compartments where biochemical and metabolic processes that regulate SA levels occur. Better understanding of SA biosynthesis and metabolism may, in the future, provide the tools necessary to manipulate SA levels, thereby enhancing plant resistance to pathogens.

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