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Biochemistry of Salicylic Acid and its Role in Disease Resistance

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Salicylic acid (SA) is involved in the establishment of systemic acquired resistance (SAR) in many plants including tobacco. Considering the important role of SA in disease resistance, biosynthetic and metabolic pathways of SA in tobacco have been studied extensively. The initial step for biosynthetic pathway of SA is conversion of phenylalanine to trans-cinnamic acid, followed by decarboxylation of trans-cinnamic acid to benzoic acid and its subsequent ring hydroxylation at the C-2 position to form SA. In TMV inoculated tobacco, most of the newly synthesized SA is glucosylated or methylated. Methyl salicylate has been identified as a biologically active, volatile signal. In contrast, the two glucosylated forms accumulate in the vicinity of lesions and consist of SA glucoside, a major metabolite, and SA glucose ester, a relatively minor form. Two enzymes involved in SA biosynthesis and metabolism have been purified and characterized : benzoic acid 2-hydroxylase which catalyzes conversion of benzoic acid to SA; UDP-Glucose: SA 1-O-D glucosyltransferase which converts SA to SA glucose ester. Further studies of the biosynthetic and metabolic pathways of SA will help to elucidate the SAR signal transduction pathway and provide potential tools for the manipulation of disease resistance.

In incompatible interactions between plants and necrotic pathogens, the spread of fungal, bacterial and viral pathogens is restricted to the necrotic area of initial infection, called hypersensitive response (HR). This HR is characterized by rapid, localized cell death, generation of active oxygen species, deposition of callose and lignin-related compounds, the production of phytoalexins, and induction of the pathogenesis-related (PR) proteins. This defense response however is not only restricted to infected tissues. Upper parts of uninfected tissues become resistant to the subsequent inoculation with other pathogens such as viruses, fungi and bacteria. This phenomenon termed systemic acquired resistance (SAR) was first described in tobacco plants infected with the tobacco mosaic virus (TMV) (Ross, 1961).

Recently, marker genes for SAR have been isolated and identified. Induction of these genes is associated with the development and maintenance of SAR. In fact, many SAR proteins belong to the class of PR proteins (Gianinazzi et al., 1970:.Van Loon and Van Kammen, 1970: Van Loon, 1985). The first PR proteins to be identified were isolated from TMV infected tobacco in which nine families of PR proteins can be currently classified as SAR markers. However, relative expression levels of SAR marker genes vary between different plant species. For example, tobacco and Arabidopsis PR-1 is strongly induced by TMV, whereas the expression of cucumber PR-1 is relatively weak (Ryals et al., 1992). Among the SAR genes, PR-2 and PR-3 showed in vitro antimicrobial activity of β-1,3-glucanase and chitinase, respectively (Ponstein et al., 1994: Schlumbaum et al., 1986). In addition, *in vitro* antimicrobial activity has been demonstrated for PR-1, PR-4 and PR-5 (Sandoz, 1991: Ponstein et al., 1994: Woloshuk et al., 1991). These data suggest that SAR proteins may function in onset of SAR or disease resistance.

Salicylic acid (SA) has been found as a naturally occurring phenolic compound in wide range of plants (Raskin, 1992). Its effects on plants have been extensively studied. Applications of SA cause physiological and biochemical changes in plants such as stimulation of flowering in the *Lemnaceae* (Cleland and Ajami, 1974: Hew, 1987), inhibition of ethylene biosynthesis (Leslie and Romani, 1986), stomatal closure (Rai et al., 1986), and ion uptake (Glass, 1973: Glass, 1974). The first demonstration of role of endogenous SA in plant development was carried out in thermogenic plants (Raskin et al., 1987). Subsequently, endogenous SA has been shown to be an important component in plant disease resistance (Malamy et al., 1990: Metraux et al., 1990).

Given the significance of SA in plant disease resistance, the pathway of SA biosynthesis and catabolism is the key regulatory point in plant disease resistance. In this review, we

examine the role of SA in SAR signal transduction and discuss recent progress in studying biosynthesis and metabolism of SA.

Role of SA and its conjugates in disease resistance

Many lines of evidence implicate SA in plant disease resistance. Early studies showed that the application of SA induced resistance to pathogens and promoted the accumulation of PR proteins (White, 1979). Later, it was shown that TMV infection resulted in accumulation of endogenous SA and the onset of SAR in the TMV resistant tobacco cultivar. However, no change in SA concentration was observed in the TMV-susceptible cultivar after TMV infection (Malamy, 1992). Furthermore, temperature-shift experiments showed that the incubation of tobacco plants at or above 32° C blocked production of SA and induction of PR proteins, which resulted in the systemic spread of TMV throughout the plant. However, temperature shift of the same plants to normal temperature (24° C) leads to an increase in the biosynthesis of SA and the expression of PR proteins. The correlation of SA accumulation and onset of SAR has been found not only in tobacco, but also in other plants such as cucumber and Arabidopsis (Malamy et al., 1990: Metraux et al., 1990: Rasmussen et al., 1991: Dempsey et al., 1993). Further evidence for the importance of SA in SAR came from studies on transgenic tobacco and Arabidopsis overexpressing SA hydroxylases (NahG) which convert SA to catechol (Gaffney et al., 1993: Delaney et al., 1994). Upon pathogen infection, such transgenic plants were unable to accumulate SA and to display the SAR response to viral, fungal or bacterial pathogens. These findings suggest that SA accumulation is necessary for the establishment of SAR.

The development of SAR requires the translocation of the signaling molecules from initial infected tissues displaying the hypersensitive response to the upper healthy leaves. It has been speculated that SA may act as a long distance signal for SAR, since SA appears SA in the phloem of both tobacco and cucumber after inoculation of pathogens. The concentration of SA also increases in upper uninfected tissues developing SAR as a result of the infection of lower leaves (Metraux et al., 1990: Yalpani et al., 1991). Two direct lines of evidence supported the hypothesis that SA acts as a long distance signal. In tobacco, 70% (^{18}O labeled SA) of SA in upper uninoculated leaves was synthesized and transported from TMV inoculated leaves (Shulaev et al., 1995). In other experiments with cucumber fed ^{14}C labeled benzoic acid, 50% of SA in upper leaves resulted from translocation of SA from tobacco necrosis virus (TNV) infected leaves to uninfected leaves (Molders et al., 1996). However, other studies suggest that SA is not the long distance signal. In cucumber, the detachment of the infected leaf before SA accumulation did not affect the increase of SA and induction of PR gene expression (Rasmussen et al., 1991). Grafting experiments showed that TMV infected transgenic (NahG) root stocks, which were incapable of accumulation of SA, were able to induce SAR and PR genes (Vernooij et al., 1994). Therefore, it is not clear if there are signals other than SA involved in the establishment of SAR or if a small amount of translocated SA is sufficient for onset of SAR.

Methyl salicylate may function as an airborne signal for both intra and inter plant communication. Treatment of tobacco plants with gaseous methyl salicylate resulted in an increase in SA and PR-proteins as well as resistance to TMV (Silverman, unpublished). Recently, methyl salicylate has been shown to be the major volatile compound released after TMV-inoculation of tobacco plants (Shulaev et al., 1997). It has also demonstrated that gaseous methyl salicylate produced by TMV-inoculated tobacco plants was able to induce expression of PR genes and resistance to TMV in neighboring healthy plants.

In contrast, however, little is known about the function of SA glucoside forms in tobacco. These conjugates do not appear to be required for the induction of resistance and PR-proteins in tobacco, since they are mainly present in and around hypersensitive lesions and are rarely detected in systemically protected tissues. (Enyedi et al., 1992: Hennig et al., 1993). In addition, the absence of SA glucoside in phloem exudates makes it an unlikely candidate for the translocatable form of SA. However, the phytotoxicity of SA (over 0.1 mM) in tobacco (Raskin, unpublished) suggests that conjugation might be required for the detoxification of SA. Alternatively, SA 1-*O*-β-D-glucoside may function as a slow release storage form of SA, which maintains systemic acquired resistance over extended periods of time. Similar storage mechanisms are known for other plant hormones. For example, cytokinin β-*O*-glucoside, a hypothesized storage compound, was hydrolyzed by a specific β-glucosidase to release active cytokinin (Brzobohaty et al., 1993). The formation of SA glucosides could also represent a route of SA catabolism. Even though the function of SA conjugates have not been defined, they are likely to serve, to some degree, in each of these capacities.

Biosynthesis of SA

The formation of *t*-cinnamic acid from phenylalanine is the starting point for the biosynthetic pathway of SA. This reaction is catalyzed by phenylalanine ammonia lyase (PAL) which is a key regulatory enzyme of the phenylpropanoid pathway. There are two plausible pathways for conversion of t-cinnamic acid to SA. The C-2 position of aromatic ring can be hydroxylated before or after chain-shortening reactions. Tomato seedlings infected with *Agrobacterium tumefaciens* accumulated SA via *o*-coumaric acid while benzoic acid is a favorable precursor for SA in noninfected plant (Chadha and Brown, 1974). The other evidence for the *o*-coumaric acid pathway showed that both radioactive *o*-coumaric acid and SA were formed in leaves of *Gaultheria procumbens* and/ or *Primula acaulis* fed [^{14}C]-labeled cinnamic acid or phenylalanine (El-Basyouni et al.,1964: Grisebach and Vollmer, 1963). These plants as well as potato tubers, *Helianthus annuus*, *Solanum tuberosum* and *Pisum sativum produced radioactive* SA after treatment with [^{14}C]-labeled benzoic acid (El-Basyouni et al., 1964: Klambt, 1962: Ellis and Amrhein, 1971). Rice seedlings also produced SA from t-cinnamic acid via benzoic acid (Silverman et al., 1995). Moreover, ^{14}C-labeling studies with cell suspensions of tobacco, and healthy and tobacco mosaic virus (TMV)-inoculated tobacco leaves demonstrated that the conversion of t-cinnamic acid to SA proceeded via decarboxylation of t-cinnamic acid to benzoic acid, followed by 2-hydroxylation of aromatic ring to SA (see Fig. 1). In these experiments, [^{14}C]-Labeled *o*-coumaric acid was not detected after treatment with labeled cinnamic acid (Yalpani et al., 1993). In summary, it seems that biosynthetic pathway of SA via benzoic acid is the predominant in most plants.

The intermediate steps in the formation of benzoic acid from *t*-cinnamic acid has not yet been elucidated. Both a nonoxidative pathway and β-oxidation pathway have been proposed for this side-chain shortening reaction. The β-oxidation of *t*-cinnamic acid to benzoic acid may occur in a manner analogous to that observed in fatty acid catabolism. The supporting evidence for this idea came from studies on cell-free extracts of *Quercus pedunculata* where ATP and acetyl-CoA stimulated conversion of cinnamic acid to benzoic acid (Alibert and Ranjeva, 1971). However, a non-oxidative mechanism has been suggested by other studies. For example, cell suspension cultures of *Vanilla planifolia* (Funk and Brodelius, 1990), *Lithospermum erythrorizum* (Yazaki et al., 1991) and Daucus carota (Schnitzler et al., 1992)

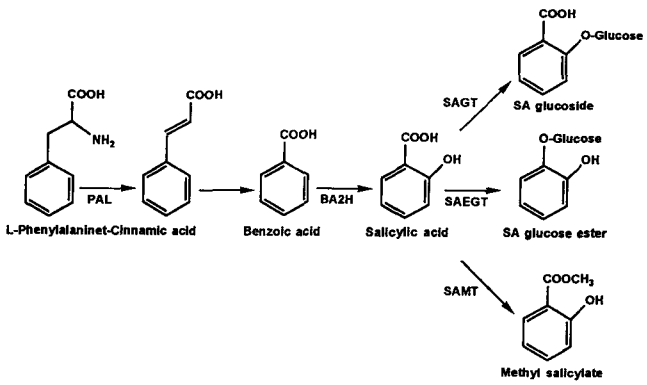

Figure 1. Schematic diagram of biosynthetic and metabolic pathway of SA in tobacco.

accumulated *p*-hydroxybenzoic acid from *p*-coumaric acid via *p*-hydroxybenzaldehyde as an intermediate.

Benzoic acid 2-hydroxylase (BA2H), a monooxygenase which belongs to the cytochrome P450 superfamily, has been identified in cell-free tobacco leaf extracts (León et al., 1993a). This enzyme specifically catalyzes the formation of SA from benzoic acid. Induction of this enzyme activity was detected in parallel with benzoic acid accumulation after TMV inoculation of tobacco plants (Yalpani et al., 1993: Leon et al., 1993a). In addition, exogenously benzoic acid induced BA2H activity in healthy tobacco plants (León et al., 1993a: León et al., 1993b). Moreover, BA2H activity increased in tobacco plants treated with UV light and ozone which also caused a significant production of active oxygen species (Yalpani et al., 1994). Interestingly, hydrogen peroxide caused rapid activation of the BA2H activity in tobacco leaves and in cell-free extracts (León et al., 1995). Unlike other eucaryotic P450 enzymes which are membrane-bound and possess molecular mass between 50-60 kD, this enzyme is soluble and has a high molecular mass of approximately 160 kD (León et al., 1995).

Metabolism of SA

Like other hydroxybenzoic acids which have been found as different types of conjugates in a variety of plant species (Pierpoint, 1994), SA is modified to form conjugates, mostly by glucosylation or less frequently by esterfication (Klick and Herrmann, 1988a). Early studies showed accumulation of trace amounts of SA and large amounts of SA glucosides in *Helianthus annuus* hypocotyls fed with ^{14}C-labeled benzoic acid (BA) (Klambt, 1962). It has been shown that SA 2-*O*-β-D-glucoside (Fig. 1) is a major SA metabolite in many

plant species including *Mallotus japonicus* (Tanaka et al., 1990: Umetani et al., 1990), spice plants (Klick and Herrmann, 1988b: Klick and Herrmann, 1988c: Herrmann, 1989), oat (Yalpani et al., 1992a: Yalpani et al., 1992b) and bean roots (Schulz et al.: 1993). Interestingly, after TMV inoculation of tobacco leaves, most of the endogenously synthesized SA is converted into the SA glucoside (Enyedi et al., 1992: Malamy et al., 1992). Although SA 2-*O*-β-D glucoside is predominant in a wide range of plants, SA also forms other metabolites by esterification, additional hydroxylation of the aromatic ring or by methylation. For example, glucose esters of SA were found in soybean cell cultures fed with ^{14}C-labeled SA or benzoic acid (Barz et al., 1978). It is interesting to note that exogenous SA can be metabolized to 2,5-dihydroxybenzoic acid (gentisic acid) and 2,3-dihydroxybenzoic acid (*O*-pyrocatechuic acid) in human. Therefore, it is not surprising that 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were detected in leaves of various plants fed radioactive SA (Ibrahim and Towers, 1959) and in *Astilbe sinensis* and tomato plants incubated with ^{14}C cinnamic and benzoic acids (Chadha and Brown, 1974: Billek and Schmock, 1967). However, it is unclear if these dihydroxybenzoic acids were present as glucoside forms, because of acid hydrolysis of samples before analysis which would have promoted their release. In roots of buckwheat (*Fagopyrum esculentum*), SA was 5-hydroxylated to 2,5-dihydroxybenzoic acid (gentisic acid), followed by glucosylation at hydroxyl group of C5-position to form gentisic acid 5-*O*-β-D-glucoside (Schulz et al., 1993). Like other plant hormones that form various metabolic products including amino acid conjugates (Reinecke and Bandurski, 1987), an amino acid conjugate form of SA, N-salicyloyl aspartic acid, was found in plants including wild grapes (*Vitis riparia, Vitis rupestris*) (Steffan et al., 1988) and French beans (Bourne et al., 1991). However, it has not been determined whether this compound is a SA metabolite or whether it forms from other intermediates of SA biosynthesis. Interestingly, only one identified SA metabolite, methyl salicylate, is volatile (Fig. 1). This SA ester is the primary constituent of the oil of wintergreen (Cauthen and Hester, 1989). This compound was also detected in other plants (Buttery et al., 1982: Buttery et al., 1984: Buttery et al., 1986: Andersen et al., 1988) and fruits (Herrmann, 1990). Recently, it was discovered that large amounts of volatile methyl salicylate were released from TMV-inoculated tobacco where SA accumulated (Shulaev et al., 1997). While there were no detectable levels of methyl salicylate in healthy or mechanically wounded tobacco leaves.

In TMV-inoculated tobacco leaves, two glucosylated forms (up to 80% of total SA) accumulated in and around necrotic lesions. SA 2-*O*-β-D-glucoside was detected as a major metabolite, while SA 1-*O*-β-D-glucoside seems to be a minor form. Phloem exudates and uninoculated leaves of TMV-inoculated tobacco contained only small amounts of glucosylated conjugates (Enyedi et al., 1992). Now, it is evident that both endogenously produced and exogenously supplied SA in tobacco is metabolized to SA 1-*O*-β-D-glucoside and 2-*O*-β-D-glucoside. In SA infiltrated tobacco, SA 1-*O*-β-D-glucoside was formed rapidly and remained at constant levels for at least 24 hour after SA treatment. In contrast, 2-*O*-β-D-glucoside accumulated slowly after SA infiltration. Our *in vitro* and in vivo data suggest that high concentrations of free SA triggers rapid formation of SA 1-*O*-β-D-glucoside and a slower formation of SA 2-*O*-β-D-glucoside, which may be a more stable metabolite of SA (Lee and Raskin, unpublished data). Recent feeding experiment in tobacco showed that release of $^{14}CO_2$ was coincident with accumulation of SA glucoside (Edwards, 1994). This experiment suggests that glucosylation of SA may be an initial step in the general catabolism.

Conversion of SA to 2-*O*-β-D-glucoside is catalyzed by UDP-glucose: SA 2-*O*-β-D-glucosyltransferase (SAGT). SAGT activity was detected in cell cultures of *Melilotus japonicus* (Tanaka et al., 1990), oat roots (Yalpani et al., 1992a: Yalpani et al., 1992b), and tobacco leaves (Enyedi and Raskin, 1993) after SA application. In TMV-inoculated tobacco, an increase in SAGT activity coincided with the accumulation of SA and the formation of its product, SA 2-*O*-β-D-glucoside. The highest enzymatic activity was localized in the vicinity of hypersensitive lesions where 2-*O*-β-D-glucoside accumulated. Thus, the spatial and temporal distribution of SAGT correlated with that of SA 2-*O*-β-D-glucoside. SAGT was partially purified and characterized in oat roots (Yalpani et al., 1992a). SA induction of tobacco and oat SAGTs are inhibited by cycloheximide, a protein synthesis inhibitor (Yalpani et al., 1992b: Enyedi and Raskin, 1993), and by RNA synthesis inhibitors in cell suspension cultures of *Melilotus japonicus* and in oat roots (Tanaka et al., 1990: Yalpani et al., 1992b). These results suggest that the induction of SAGT by SA is regulated at the transcriptional level. Recently, Lee and Raskin have purified and characterized biochemical properties of UDP-glucose: SA 2-*O*-β-D-glucosyltransferase (SAEGT) which is responsible for conversion of SA to 2-*O*-β-D-glucose (Lee and Raskin,

unpublished data). The molecular mass of tobacco SAEGT is approximately 48 kD which is in agreement with molecular masses of other known glucosyltransferases (GTs) which range between 40 and 60 kD. This enzyme utilized only UDP-glucose as a sugar donor and, like other GTs, was strongly inhibited by UDP (Lee and Raskin, unpublished data). Previously identified GTs are either localized in the cytoplasm or are associated with cell membrane. In the case of SAEGT, the enzyme activity was found in the soluble protein fraction, suggesting that SAEGT is a cytoplasmic enzyme (Lee and Raskin, unpublished data).

Future direction

SA is an important component of plant disease resistance but many unanswered questions remain concerning its precise role(s). What is the mode of SA action in SAR? Is SA a primary long distance signal in SAR? If not, what is that molecule? How is SA biosynthesis activated by pathogens? Which steps of SA biosynthetic and metabolic pathways are rate limiting? It is also not clear if SA biosynthesis and metabolism varies in different plants. Identification and characterization of enzymes and genes involved in SA biosynthesis and metabolism will help to address these questions.

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