Physiologia Plantarum 129: 461–471. 2007

A new catalytic activity from tobacco converting 2-coumaric acid to salicylic aldehyde

Jacek Malinowski[a,*], Magdalena Krzymowska[b], Kamil Godoń[a], Jacek Hennig[b] and Andrzej Podstolski[a]

[a]Department of Plant Growth and Development, Institute of Experimental Plant Biology, Faculty of Biology, Warsaw University, Miecznikowa 1, 02-096 Warsaw, Poland

[b]Plant Pathogenesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5A, 02-106 Warsaw, Poland

Correspondence
*Corresponding author,
e-mail: jacmalin@biol.uw.edu.pl

Received 28 August 2006; revised 14 September 2006

doi: 10.1111/j.1399-3054.2006.00837.x

Salicylic acid (SA) mediates plant response to pathogen invasion, resulting in hypersensitive response and in the formation of systemic acquired resistance. It is well known that *Nicotiana tabacum* and other plants respond to Tobacco Mosaic Virus (TMV) infection by increasing the content of SA but the details of SA biosynthesis are still not fully understood. Generally, SA may originate directly from isochorismate (*Arabidopsis thaliana*), or its C_6–C_1 skeleton could be synthesized via the phenylpropanoid pathway by β-oxidation of *trans*-cinnamic acid (*N. tabacum*), 2-coumaric acid (OCA) (*Gaulteria procumbens*, *Lycopersicum esculentum*) or by retro-aldol reaction of *trans*-cinnamoyl-CoA (*Hypericum androsaemum*). We report here a novel putative enzyme activity from tobacco, salicylic aldehyde synthase (SAS), catalysing non-oxidative formation of salicylic aldehyde (SALD) directly from OCA. This chain-shortening activity is similar to that of 4-hydroxybenzaldehyde synthase from *Vanilla planifolia*, *Lithospermum erythrorhizon*, *Daucus carota*, *Solanum tuberosum* and *Polyporus hispidus* but the enzyme differs in the kinetics of the reaction, substrate specificity and requirements for reducing cofactors. SAS activity is constitutively expressed in healthy tobacco leaves and doubles as a result of infection with TMV. Moreover, the product of SAS activity—SALD, applied exogenously on tobacco leaves, stimulates peroxidase activity and enhances resistance to consecutive infection with TMV. These observations could suggest a contribution of SAS and SALD to the response of tobacco to TMV infection.

Introduction

Among the plant secondary products, C_6–C_1 metabolites are ascribed a particularly important role. This group of compounds, as well as their derivatives, participates in allelopathy (Walker et al. 2003, Wu et al. 2002), airborne signal transmission between neighbouring plants (Shulaev et al. 1997), attract pollinators (Dudareva et al. 2000) and carnivorous insects (Van Poecke and Dicke 2002, Van Poecke et al. 2001). They are also involved in photoperiodic regulation of flowering (Cleland and Ajami 1974, Martínez et al. 2004). Benzoic acid (BA) derivatives can serve as intermediates in ubiquinone and

Abbreviations – 4-HBALD, 4-hydroxybenzaldehyde; 4-HBS, 4-hydroxybenzaldehyde synthase; AS, ammonium sulphate; BA, benzoic acid; BA-2-OH, benzoic acid 2-hydroxylase; BD, benzaldehyde; CL, coenzyme A ligase; GCMS, gas chromatography mass spectrometry; HPLC, high-performance liquid chromatography; HR, hypersensitive response; ICS, isochorismate synthase; OCA, 2-coumaric acid, 2-hydroxycinnamic acid; SA, salicylic acid, 2-hydroxybenzoic acid; SALD, salicylic aldehyde, 2-hydroxybenzaldehyde; SALDDH, salicylic aldehyde dehydrogenase; SAR, systemic acquired resistance; SAS, salicylic aldehyde synthase; TMV, Tobacco Mosaic Virus; TCA-2-OH, *trans*-cinnamic acid 2-hydroxylase.

plastoquinone biosyntheses (Bell and Charlwood 1980), and are precursors or constituents of pharmaceuticals—such as paclitaxel (Chu et al. 1994), cocaine (Bjorklund and Leete 1992), shikonine (Lösher and Heide 1994) and flavour compounds: vanillin, vanillic acid and 4-hydroxybenzaldehyde (4-HBALD) (Funk and Brodelius 1990).

To defend against pathogens, among other mechanisms, plants have developed hypersensitive response (HR) and systemic acquired resistance (SAR). HR leads to the formation of local necrotic lesions, restricting spreading of the pathogen. The primary infection that triggers HR may also result in the development of SAR, a long-lasting resistance to secondary infection, developed in the whole plant (Ross 1961, Ryals et al. 1996). Both these responses are associated with production of SA adjacent to the infection site and in systemic parts of the plant (Enyedi et al. 1992, Malamy et al. 1990, Métraux et al. 1990, Yalpani et al. 1991). Malamy and co-workers have shown that in tobacco infected with Tobacco Mosaic Virus (TMV) local concentration of SA raises quickly and reaches its maximum 72 h after inoculation. In parallel, an induction of PR-1 (pathogenesis-related protein) gene expression was observed. Experiments with transgenic *nahG* tobacco plants expressing a bacterial gene for salicylic acid (SA) hydrolase showed that such plants were unable to accumulate SA and also could not establish SAR in response to primary infection (Gaffney et al. 1993). The central role of SA in regulation of HR and SAR after pathogen infection is well established (Delaney et al. 1994, Lawton et al. 1995, Ryals et al. 1995, Venooij et al. 1994).

Plants can communicate with each other by transmitting a complex mixture of volatile substances through the air (Arimura et al. 2000, Baldwin and Schultz 1983). Among them are derivatives of fatty acids, ethylene, terpenoids and phenolics (Arimura et al. 2001, Engelbert et al. 2004, Farmer and Ryan 1990). In tobacco, the volatile methyl salicylate has been shown to be released from leaves inoculated with TMV and postulated to be responsible for systemic activation of defence-related genes in tissues remote from the place of the infection and even in neighbouring plants (Shulaev et al. 1997). It seemed of interest whether any other volatile phenolic compound—e.g. salicylic aldehyde (SALD)—might also serve as a volatile airborne signal involved in the plant–plant cross-talking.

Despite numerous investigations, the biosynthesis of SA in plants is not fully understood. Two main pathways of SA biosynthesis are postulated: the phenylpropanoid pathway in tobacco (Yalpani et al. 1993) and the chorismate pathway described in *Arabidopsis thaliana* (Wildermuth et al. 2001). Both await the complete characterization of the participating enzymes. SA biosynthesis via the phenylpropanoid pathway has to involve a step of side chain shortening of cinnamic acid by two carbons. The mechanism of this reaction may be oxidative or non-oxidative (Fig. 1). The first one requires formation of *trans*-cinnamoyl-CoA, followed by its degradation to BA according to the scheme of β-oxidation of fatty acids (Zenk 1965). The BA is next hydroxylated in position 2 to form SA (Fig. 1). In fact, tracer studies with radioactive *trans*-cinnamic acid showed that the label moves from it to SA via BA in mock- and TMV-inoculated leaves (Yalpani et al. 1993). However, when radiolabelled BA was administered, the amount of SA in TMV-inoculated plants was higher than the amount of the labelled precursor supplied and a dilution of radioactivity was observed. This suggested that after TMV infection a pathway of SA biosynthesis, different than via BA was engaged. At the same time the activity of benzoic acid 2-hydroxylase was detected in healthy tobacco leaves, which was significantly increased upon TMV infection (Leon et al. 1993).

It has been postulated that the β-oxidative pathway leading to SA can also utilize 2-coumaric acid (OCA) as a substrate. According to this hypothesis active CoA ester of OCA undergoes oxidative chain shortening, forming directly SA (Fig. 1). Such reactions were reported in *Gaulteria procumbens* (El-Basyouni et al. 1964), and tomato seedlings infected with *Agrobacterium tumefaciens* (Chadha and Brown 1974). However, experiments of Yalpani et al. (1993) negated that OCA could be a substrate of SA biosynthesis in tobacco, as they observed that application of OCA to tobacco did not promote SA accumulation.

The second—non-oxidative—pathway leads to the formation of benzaldehydes (BD), possibly oxidized further to corresponding acids by aldehyde dehydrogenase (Fig. 1). Such a process has been observed in *Daucus carota* suspension cultures, where 4-hydroxycinnamic acid was non-oxidatively converted to 4-HBALD and subsequently oxidized to 4-hydroxybenzoic acid by nicotinamide adenine dinucleotide-dependent aldehyde dehydrogenase (Schnitzler et al. 1992). These two pathways could be linked by a retro-aldol reaction, similar to that reported in *Hypericum androsaemum* (El-Mawla and Beerhues 2002) where CoA esters were degraded to corresponding aldehydes in a non-oxidative manner. The findings of Ribnicky et al. (1998) showed that application of BD to tobacco plants caused a 13-fold increase in SA concentration, and after application of radioactive [$^{13}C_6$]BD to leaves the label moved to SA via BA. At the same time, application of radiolabelled *trans*-cinnamic acid showed that no label was incorporated into BD. Later, BD indeed was proven not to be involved in the biosynthesis of SA from *trans*-cinnamic acid in tobacco (Jarvis et al. 2000). So, in the light of feeding experiments

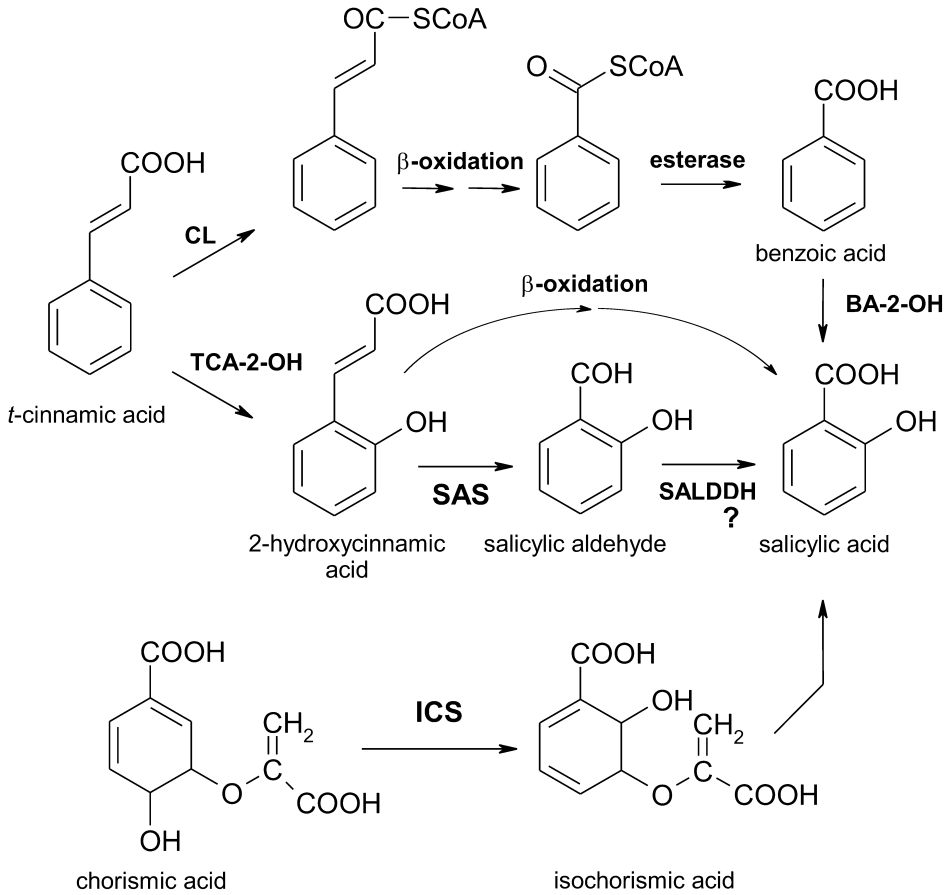

Fig. 1. Possible routes of salicylic acid biosynthesis in plants via the chorismic acid pathway and the phenylpropanoid pathway with oxidative or non-oxidative chain shortening of cinnamic acid. CL, coenzyme A ligase; BA-2-OH, benzoic acid 2-hydroxylase; ICS, isochorismate synthase; SALDDH, hypothetic salicylic aldehyde dehydrogenase; TCA-2-OH, *trans*-cinnamic acid 2-hydroxylase.

with radioactive BD, its participation in SA biosynthesis remains a mystery.

On the other hand, not much is known about another potential precursor of SA—SALD. In this article we describe a novel activity of a putative enzyme observed in tobacco leaves—salicylic aldehyde synthase (SAS) catalysing non-oxidative transformation of OCA (2-hydroxycinnamic acid) directly (Fig. 1) to SALD.

Materials and methods

Plant material

Wild-type and transgenic nahG (Syngenta Biotechnology Inc., Durham, NC) *Nicotiana tabacum* cv. Xanthi nc plants were cultivated in a phytotrone chamber under 16-h photoperiod at 22°C on peat substrate (Degernes Agro-Plast, Denmark). Light conditions were 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (daylight and warm white 1:1, LF-40W; Pila, Poland).

Preparation of crude and partially purified SAS extract

Fully developed leaves of 8-week-old tobacco plants were harvested without main veins. The material (2.5 g) was placed in 5 ml of 50 m*M* Tris–HCl buffer, pH 7.7 containing 10 m*M* β-mercaptoethanol, 0.05% sodium dodecyl sulphate, 10% w/v polyvinylpolypyrrolidone (PVPP) (Polyclar AT, Serva Electrophoresis GMBH, Germany) and homogenized in an Ultra Turrax T25 homogenizer (Janke and Kunkel, GmbH & Co. KG—IKA-Labortechnik, Germany) 3 × 15 s at 24 000 rpm. The mixture was centrifuged at 20 000 *g* for 30 min at 4°C and 3 ml of the supernatant was freed of low molecular substances by filtration through an Econo-Pac 10DG (Bio-Rad, Hercules, CA) column equilibrated with 50 m*M* Tris–HCl buffer pH 7.7, according to the manufacturer's protocol. The filtrate was used as the SAS crude extract. Protein concentration was estimated according to the method of Bradford (1976). To obtain partially purified SAS extract, solid ammonium sulphate (AS) was added to

the crude extract to 40% of saturation and the mixture was incubated for 30 min at 4°C. After centrifugation 20 000 g for 30 min at 4°C), the resulting supernatant was subjected to hydrophobic interactions chromatography on a HiPrep 16/10 Phenyl FF low subcolumn (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was equilibrated at a flow rate of 2 ml min^{-1} with buffer A (12.5 m*M* pyrophosphate buffer, pH 6.5 containing AS at 40% saturation) and 20 ml of the supernatant was applied. Next an elution gradient A to B (12.5 m*M* pyrophosphate buffer, pH 6.5) was started over 45 min at a flow rate of 2 ml min^{-1}. Fractions (2 ml) of eluted protein extract were collected and SAS activity in all the fractions was assayed. Maximum peak of SAS activity was determined in fractions eluted at approximately 5% of saturation of AS. Fractions with SAS activity were pooled and freed of AS by filtration using an Amicon Ultra 15 centrifugal filter device (Millipore, Bedford, MA) with a molecular weight cut-off at 10 000, according to the manufacturer's protocol.

SAS activity assay

Crude SAS extract (100 μl) was added to 150 μl of 50 m*M* Tris–HCl buffer, pH 7.7, containing 10 m*M* cysteine, and 5 m*M* OCA as a substrate and incubated at 35°C for 10 min. The reaction time was chosen to obtain detectable amounts of the product—SALD. For characterization of the reducing agent requirements, the following additions were used: cysteine (1–20 m*M*), dithioerythritol (10 m*M*), dithiothreitol (10 m*M*) β-mercaptoethanol (10 m*M*), glutathione in reduced form (10 m*M*), glutathione in oxidized form (10 m*M*) and coenzyme A (10 m*M*). For characterization of substrate specificity, 5 m*M* OCA, 4-hydroxycinnamic acid, 4-methoxycinnamic acid, *trans*-cinnamic acid, caffeic acid, ferulic acid and sinapic acid were used. To determine the linearity of the reaction with time we used partially purified SAS protein extract, 5 m*M* OCA as a substrate and 10 m*M* of cysteine. The reaction times were 0, 1, 2, 5 and 10 min. The reaction was stopped by adding 125 μl of a mixture containing 10% glacial acetic acid and 90% methanol. After that samples were frozen (30 min at −80°C), thawed and centrifuged at 10 000 *g* for 15 min. The resulting supernatants were analysed by high-performance liquid chromatography (HPLC).

HPLC analysis

Products of SAS activity were analysed by HPLC (LC 1120 isocratic pump, GBS, Australia; UV monitor, SPD-10A*VP* UV–VIS detector, Shimadzu, Japan), on a C-18 RP column (Luna 3 μm, 150 mm × 2.00 mm, Phenomenex, Torrance, CA). The solvents and detection wavelengths used were as follows. For 2-coumaric, 4-methoxycinnamic and *trans*-cinnamic acids, 24% acetonitrile, 25 m*M* sodium acetate and 5% glacial acetic acid in water, corresponding products of the reactions (SALD, 4-methoxybenzaldehyde, BD) were detected at 256, 284 and 247 nm, respectively. For 4-hydroxycinnamic and ferulic acids, 15% acetonitrile and 2% glacial acetic acid in water and corresponding products of the reactions (4-HBALD, vanillin) were detected at 284 and 278 nm, respectively. For sinapic and caffeic acids, 15% methanol and 2% glacial acetic acid in water and products of the reactions (3,4-dimethoxybenzaldehyde, 3,4-dihydroxybenzaldehyde) were detected at 308 and 278 nm, respectively. Comparison of the ultraviolet spectra of SALD with a commercial standard (Sigma-Aldrich, St. Louis, MO) was conducted using a Photodiode Array Monitor (Alliance System, Waters). For gas chromatography mass spectrometry (GCMS) analysis on an SPB™ 5 column (30 m × 0.25 μm, Supelco) a Hewlett Packard 5890 Series II apparatus was used. Conditions of gas chromatography run were as follows: helium flow, 1 cm min^{-1}; injector temperature, 250°C; detector temperature, 300°C. To elute the sample a temperature gradient was used: 80°C, 2 min, 80–220°C in 28 min, 220°C to 300 in 8 min.

Inoculation of tobacco with TMV

For infection experiments, 6- to 8-week-old plants were used. For inoculation with TMV, carborundum-dusted leaves were rubbed with TMV strain U1 solution (1 μg ml^{-1}) or water (mock), and next rinsed with water. Three fully expanded leaves were inoculated on each plant and after 72 h they were harvested, immediately frozen in liquid nitrogen and stored at −80°C.

Treatment of tobacco plants with SALD

Eight-week-old plants were placed in hermetic jars (6 l) and SALD was applied at a concentration of 1 or 10 μ*M* three times to each jar, in 24-h intervals. In control plants, SALD treatment was omitted. After the last application of SALD, plants to be used for analysis of peroxidase activity were incubated for 24, 72 or 120 h and subsequently the material was collected, frozen in liquid nitrogen and stored at −80°C. The plants to be used to analyse necrotic lesion sizes were incubated for the next 7 days and then inoculated with TMV, as described previously. Seven days post-inoculation

diameters of the lesions were measured by comparison with a metric standard and statistical analysis (110 lesions for SALD-treated and 110 for control plants) was conducted. The significance of the differences in lesion sizes between SALD-treated and control plants was assessed with one-way analysis of variance (ANOVA).

Measurement of peroxidase activity

Plant leaves were homogenized in 50 m*M* Tris–HCl buffer pH 7.4, containing 0.4% Triton X-100 and 2% PVPP, sonicated for 1 min at 25 W (KT 50, Walter FC Ebel, Germany) and centrifuged at 10 000 *g* for 25 min at 4°C. Supernatant (3 ml) was freed of low molecular weight substances by filtration through an Econo-Pac 10DG (Bio-Rad) column equilibrated with 50 m*M* Tris–HCl buffer pH 7.4, and used as a crude enzyme source. The reaction mixture included 0.1 *M* phosphate buffer pH 6.1, 10 m*M* guaiacol, 5 m*M* H_2O_2 and enzymatic crude extract (25 μl) in a total volume of 1 ml. The increase in absorbance was recorded at 470 nm during 60 s and peroxidase activity was expressed as μmol of synthesized tetraguaiacol per minute and mg of protein, using the millimolar extinction coefficient $\epsilon = 26.6$.

Proteins from the crude extract (25 μg) were separated on 8% polyacrylamide gels in non-denaturating conditions according to a modified Laemmli (1970) protocol. Peroxidase activity was visualised by incubation of the gels after electrophoresis in 0.1 *M* acetate buffer pH 6.0 containing 5 m*M* H_2O_2 and 1 m*M* benzidine, until bands developed.

Chemicals used

All chemicals used were purchased from Sigma-Aldrich.

Results

Properties of SAS

The extract obtained from *N. tabacum* leaves showed SAS activity, converting OCA to SALD. The reaction product was identified after HPLC separation by comparison of its peak retention time with that of a SALD standard (Fig. 2). The identity of SALD was confirmed by GCMS analysis showing no differences between the m/z lines in the mass spectra of the product and the standard (Fig. 3).

The linearity in time of the SAS-catalysed reaction was determined with partially purified (approx. 12-fold) enzymatic extract. The purification steps included AS fractionation and hydrophobic interaction chromatography. The regression coefficient R^2 calculated for the reaction times up to 10 min was 0.9988 (data not shown) confirming very strong linearity of the reaction in time.

SAS showed no distinct substrate specificity (Table 1). 4-Hydroxycinnamic acid was converted near equally well as OCA, whereas 4-methoxycinnamic and *trans*-cinnamic acids were poorer substrates. The corresponding products of the reactions were 4-HBALD, 4-methoxybenzaldehyde and BD, respectively.

The SAS Michaelis constant Km with OCA as the substrate, calculated according to the Lineweaver–Burke method, was 0.625 m*M*. The optimum of SAS activity was at pH 7.7 and at 35°C. In those optimal assay conditions, the conversion rate of OCA to SALD was 4–6 nmol of SALD per mg protein per minute.

The activity of SAS was strictly dependent on the presence of a reducing agent in the reaction mixture. We measured the SAS activity with different reductors in the assay. The highest activity was observed in the presence of 10 m*M* cysteine (Table 2).

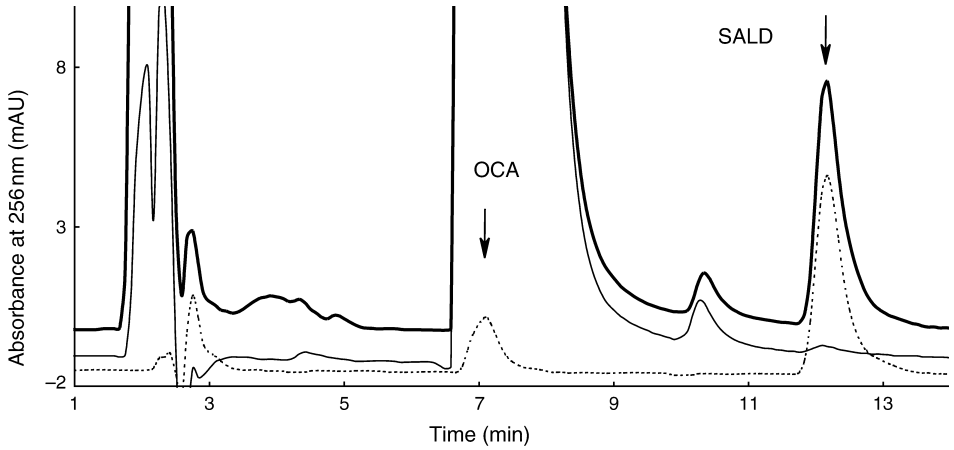

Fig. 2. High-performance liquid chromatography separation of the salicylic aldehyde synthase reaction mixture (bold line), control reaction without the enzyme (thin line) and standards [2-coumaric acid (OCA), salicylic aldehyde (SALD); dotted line]. Positions of OCA and SALD peaks are indicated.

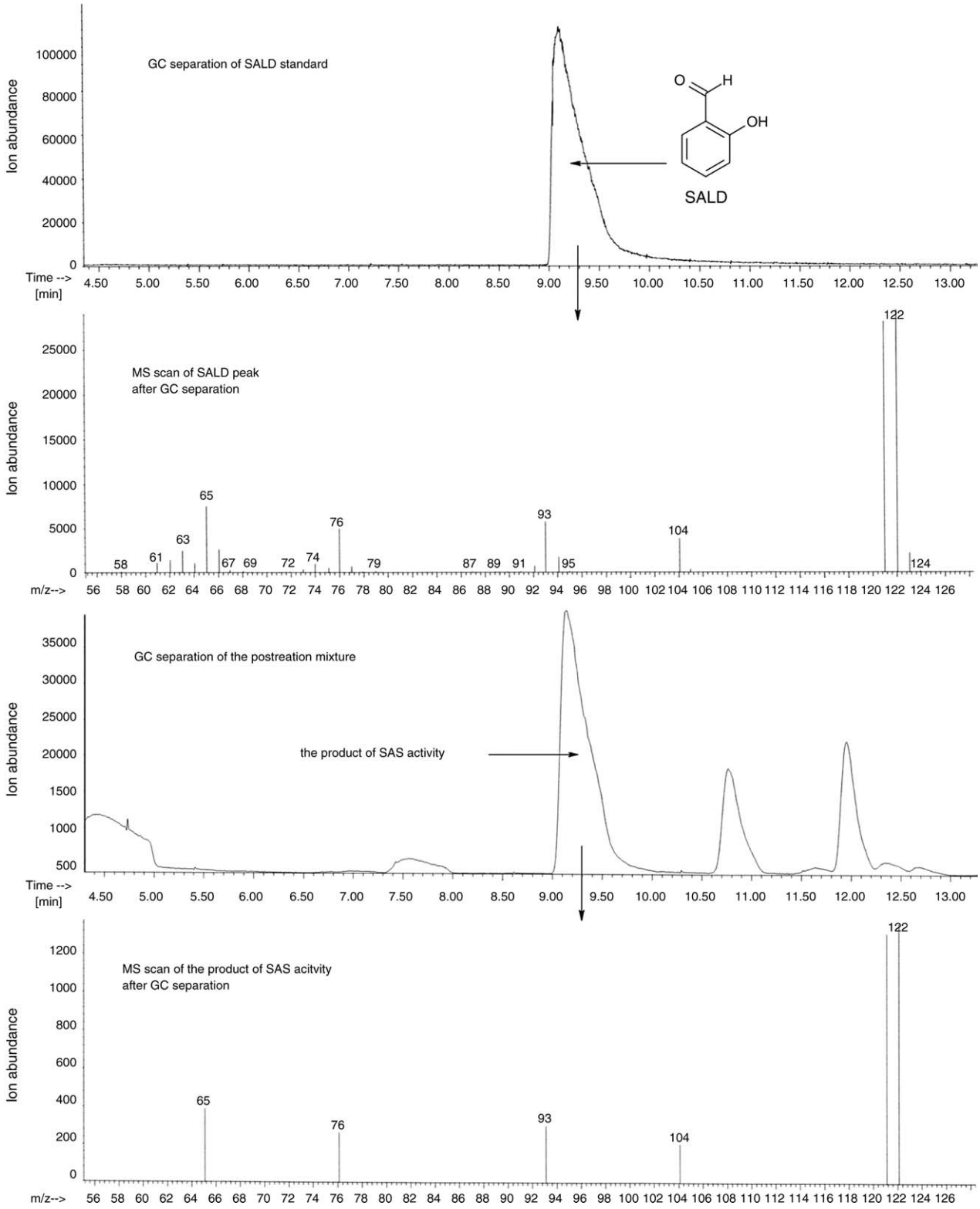

Fig. 3. Comparison of retention times and mass spectra of the product of salicylic aldehyde synthase activity after gas chromatographic separation with those of a salicylic aldehyde standard.

Table 1. Substrate specificity of salicylic aldehyde synthase (SAS) in crude extract from tobacco leaves. The standard assay contained 5 m*M* substrate, 10 m*M* cysteine, 100 μl of crude leaf extract (approx. 0.1 mg of protein) in 50 m*M* Tris–HCl, pH 7.7 in a total volume of 250 μl. Values are means ± standard deviation.

Substrate	SAS specific activity (nmol mg^{-1} protein min^{-1})	Relative activity (%)
2-Coumaric acid	5.8 ± 0.3	100
4-Hydroxycinnamic acid	5.2 ± 0.4	89
4-Methoxycinnamic acid	3.2 ± 0.1	48
trans-Cinnamic acid	1.7 ± 0.1	28
Caffeic acid	0	0
Ferulic acid	0	0
Sinapic acid	0	0

SAS activity changes after TMV infection

To determine possible contribution of SAS to the plant defence response against viral infection, we examined SAS activity in tobacco leaves 72 h after inoculation with TMV resulting in necrosis development. At that time the peak of SA concentration in inoculated leaves was reported (Malamy et al. 1990). Thus, the enzymes participating in SA biosynthesis were expected to be highly active. Both TMV- and mock-treated leaves were collected and SAS activity was assayed. TMV inoculation resulted in a two-fold higher SAS activity compared with mock-inoculated leaves (Table 3).

Treatment of plants with SALD

Peroxidases are well-known components of plant defence system (Rasmussen et al. 1995). Their important function in response to pathogen attack is lignin formation, cross-linking of the cell wall components and hydroxyperoxide level control (Bestwick et al. 1998). Treatment of tobacco plants with SALD caused an increase in peroxidase activity assayed with guiacol. After 7 days from the beginning of the experiment, plants treated with SALD showed a 1.5–2 times higher peroxidase activity than the control. This stimulation was stronger with rising concentration of SALD (Fig. 4A). However, a minor increase in peroxidase activity was also observed in leaves of untreated plants. Visualisation of peroxidase activity on polyacrylamide gel showed an increase in several peroxidase isozymes in leaves of treated plants (Fig. 4B).

Inoculation of SALD-treated and control plants with TMV resulted in the formation of necrotic lesions on leaves of both plants. Plants pretreated with SALD formed lesions smaller by ca. 20% compared with the control. ANOVA test (Table 4) proved that the observed differences showed strong statistical significance (calculated value $F = 38.9$ for $P = 2.3 \times 10^{-8}$, data not shown). To check whether SALD could be acting via SA, we conducted a similar experiment using transgenic nahG tobacco plants that are unable to accumulate SA. The necrosis sizes on leaves of SALD-treated plants were smaller by ca. 6% and the differences were statistically significant (F value = 5.9, for $P = 0.016$, according to the ANOVA test, data not shown).

Table 2. Dependence of salicylic aldehyde synthase (SAS) activity on reducing agents. The assay contained 5 m*M* 2-coumaric acid, 1–20 m*M* of the reducing agent, 100 μl of crude leaf extract (approx. 0.1 mg of protein) in 50 m*M* Tris–HCl, pH 7.7, in a total volume of 250 μl. Values are means ± standard deviation.

Reducing agent	SAS relative activity (%)
Cysteine, 20 m*M*	84.2 ± 4.1
Cysteine, 10 m*M*	100 ± 6.9
Cysteine, 5 m*M*	71.8 ± 2.0
Cysteine, 2.5 m*M*	37.8 ± 3.4
Cysteine, 1 m*M*	18.32 ± 1.5
Dithioerythritol, 10 m*M*	80.2 ± 5.3
Dithiothreitol, 10 m*M*	52.2 ± 4.9
β-Mercaptoethanol, 10 m*M*	28.1 ± 0.7
Glutathione reduced, 10 m*M*	17.7 ± 0.1
Coenzyme A, 10 m*M*	8.9 ± 1.2
Glutathione oxidized, 10 m*M*	0

Table 3. Upregulation of salicylic aldehyde synthase (SAS) activity after infection with Tobacco Mosaic Virus (TMV). In each independent experiment, three leaves from one plant were inoculated with TMV and three leaves from a control plant were water inoculated (mock). Three days after inoculation the treated leaves were harvested and SAS activity was assayed. The experiment was performed three times with similar results. Values are means ± standard deviation.

Experiment number	Variant	SAS specific activity (nmol mg^{-1} protein min^{-1})
1	Mock	4.9 ± 0.5
	TMV	9.5 ± 0.3
2	Mock	5.4 ± 0.6
	TMV	12.8 ± 0.3
3	Mock	5.8 ± 0.3
	TMV	12.3 ± 0.1

Discussion

In this work we have documented a likely enzymatic activity converting OCA into SALD in a protein extract from tobacco leaves. Our results suggest the presence of a new putative enzymatic activity—salicylic aldehyde

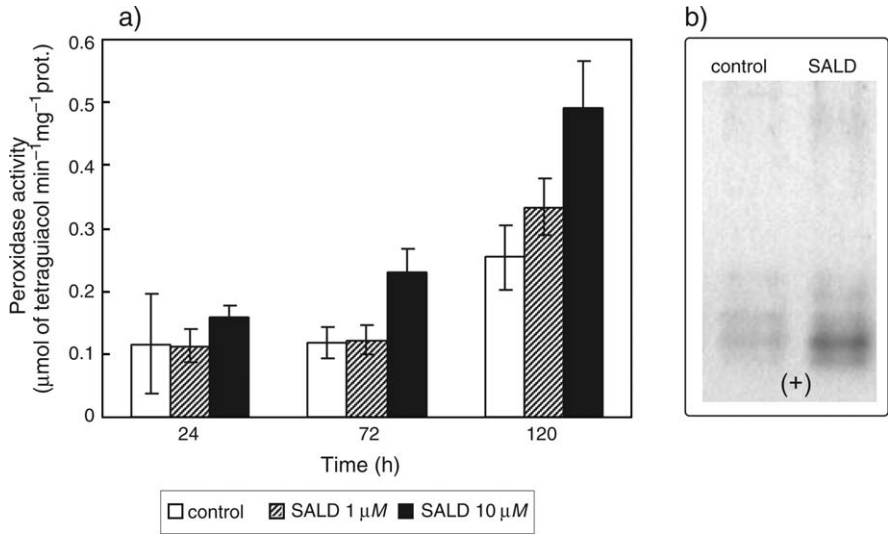

Fig. 4. Changes in peroxidase activity upon treatment of plants with salicylic aldehyde (SALD). Tobacco plants were treated with 1 or 10 μ*M* SALD (A). Visualisation of peroxidase activities with benzidine after separation on polyacrylamide gel. Extract was obtained from leaves of plants treated with 10 μ*M* SALD or control plants, 5 days after treatment. Equal amounts of protein (25 μg) were applied to the each lane (B).

synthase (SAS). The mechanism of the reaction of SALD synthesis was non-oxidative, as it proceeded in the absence of adenosine 5′-triphosphate and CoA—cofactors required for the chain shortening of cinnamic acids via the β-oxidative pathway. A similar non-oxidative mechanism was proposed for the enzymatic conversion of 4-hydroxycinnamic acid to 4-HBALD by synthases from *Vanilla planifolia* (Podstolski et al. 2002), *Lithospermum erythrorhizon* (Yazaki et al. 1991), *D. carota* (Schnitzler et al. 1992), *Solanum tuberosum* and *Polyporus hispidus* (French et al. 1976). Analysis of tobacco SAS substrate specificity showed that OCA was the substrate converted at the highest rate but 4-hydroxycinnamic and *trans*-cinnamic acids were also accepted. The corresponding products of the latter two substrates were 4-HBALD and BD, respectively. The lack of a strict substrate specificity distinguishes SAS from the chain-shortening enzyme [4-hydroxybenzaldehyde synthase (4-HBS)] from *V. planifolia* (Podstolski et al. 2002), which manifests a distinct substrate specificity for 4-hydroxycinnamic acid. But, similarly as for the vanilla enzyme, caffeic, ferulic and sinapic acids were not substrates for tobacco SAS at all. The Michaelis constant (Km) calculated for SAS with OCA was 0.625 m*M*, suggesting a rather weak affinity of OCA for the putative SAS enzyme and implicating that compounds other than OCA might be preferred by the enzyme. In our experiments, 4-HBALD and BD could also be synthesized by SAS from appropriate substrates. Both these compounds are known to be involved in plant metabolism and could also undergo further oxidations forming 4-hydroxybenzoic acid and BA, respectively. It has been shown, that in petunia a free pool of BD is present in flowers and it is also emitted into the air. BD was also demonstrated as an intermediate in non-oxidative biosynthesis of benzenoid compounds in this plant (Boatright et al. 2004). In vanilla, it is postulated that 4-H-BALD is one of the intermediates in vanillin biosynthesis (Podstolski et al. 2002).

Table 4. Effect of salicylic aldehyde (SALD) treatment on Tobacco Mosaic Virus (TMV)-induced lesions. In the experiment, three control and three SALD-pretreated plants were inoculated with TMV and after 7 days diameters of necrotic lesions were measured. The results were subjected to one-way analysis of variance and F = 38.9 for $P = 2.3 \times 10^{-8}$ was calculated, which confirms that the results are statistically significant.

Variant	No. of lesions measured	Total diameter of lesions (mm)	Mean lesion diameter (mm)	Variance (mm)
Control	110	650.6	5.9	2.14
SALD treated	110	529.9	4.8	1.77

We have shown that the best reducing agent for SAS is 10 m*M* cysteine, whereas with the same concentration of CoA the reaction proceeded at the slowest rate. The absolute requirement for the addition of a reducing agent in the assay is consistent with the data for the enzyme isolated from *V. planifolia*. However, 4-HBS from *V. planifolia* (Podstolski et al. 2002) showed the highest activity in the presence of 10 m*M* CoA and the lowest with 10 m*M* cysteine.

We observed doubling of the SAS-catalysed conversion of OCA to SALD in TMV-inoculated leaves compared with control plants. Parallely, the results of our experiments indicated that exogenous application of gaseous SALD to

wild-type tobacco plants prior to TMV inoculation decreased the diameter of necrosis lesions on the leaves by ca. 20%. It seems likely that SALD treatment reduced the spreading of TMV infection, resulted in smaller necrotic lesions. At the same time, when SA-depleted transgenic nahG plants were used in such experiment, a smaller reduction (by ca. 6%) in necrosis size was registered. It was previously shown that in nahG plants larger necrosis lesion sizes are observed than in wild-type plants after infection with TMV (Gaffney et al. 1993). Moreover, it is known that exogenous application of SA and its bioactive analogue acetylsalicylic acid to wild-type tobacco leaves reduces the size of TMV-induced necrotic lesions (White 1979). So a likely explanation of the SALD action would be its conversion to SA occurring in the leaves of the plant. However, in wild-type plants a much stronger SALD effect on necrotic lesion size was seen than in nahG plants, suggesting that the SALD action via SA could not be the only mechanism. We showed that SALD-pretreated plants exhibited an elevated peroxidase activity measured with guaiacol. It is well established that peroxidases are responsible for maintaining a proper oxidative state of the cell. This ability is shown by both ascorbate-dependent peroxidases belonging to class I and guaiacol peroxidases belonging to class III (Mehlhorn et al. 1996). Induction of the activity of class III peroxidases by SALD treatment possibly enhances the tolerance of the oxygen stress that occurs during the oxidative burst accompanying the HR response of tobacco to TMV. In this context, removing the excessive amounts of reactive oxygen species by antioxidative enzymes may result in a smaller damage to the tissue surrounding the site of infection and lower the size of the necrotic lesions formed. This may implicate an involvement of SALD formed by SAS in the plant response to the pathogen challenge. However, for the time being this hypothesis is highly speculative and needs further investigation.

SALD would be a good substrate in SA biosynthesis, involving an as yet unidentified dehydrogenase such as that reported in carrot suspension culture (Schnitzler et al. 1992) and tobacco pollen (Op den Camp and Kuhlemeier 1997), although the experiments of Yalpani et al. (1993) with application of possible SA precursors to tobacco leaf discs showed no promotion of SA biosynthesis by OCA. Thus, the participation of SAS in SA biosynthesis remains uncertain. However, our experiments with nahG plants support the hypothesis that exogenously applied SALD could to some extent be converted into SA.

It seems certain that in plants more than one pathway of SA biosynthesis is active. Wildermuth et al. (2001) revealed that mutants of *A. thaliana* with inactive isochorismate synthase (ICS) can still accumulate low levels of SA, suggesting that SA could partially originate from the phenylpropanoid pathway. On the other hand, until now, no ICS contribution to SA biosynthesis has been reported in tobacco, so in this plant SA should be obtained, at least partially, from phenylpropanoids. Additionally, it is also postulated that the intermediates of SA biosynthesis are likely glucosylated (Chong et al. 2001). All in all, the details of SA biosynthesis in tobacco remain unknown.

Volatile phenolic compounds serve as signals generated during various biotic stresses (Baldwin and Schultz 1983, Chen et al. 2003, Kessler and Baldwin 2001, Lee et al. 1995). We expect that SALD produced by SAS from OCA would also be a good airborne signal candidate released from leaves after a pathogen attack, training plants for pathogen challenge. However, emission of SALD from tobacco leaves still needs to be documented.

SAS activity is constitutively expressed in healthy tobacco leaves, therefore it is likely that production of SALD is controlled not only by SAS activity but by the substrate accessibility as well. Unfortunately, no quantitative data are available regarding the presence of OCA in tobacco leaves. However, Yalpani et al. (1993) identified OCA (below 2.5 $\mu g\ g^{-1}$ fresh weight) in TMV-inoculated leaves following temperature shift from 32 to 24°C. Also in *A. thaliana* root exudates allelopathic amounts of OCA were detected after eliciting with jasmonic acid and treatment with the fungal pathogen *Rhizoctonia solani* (Walker et al. 2003). It is possible that in tobacco the biosynthesis of OCA may proceed by 2-hydroxylation of *trans*-cinnamic acid in a reaction catalysed by a hydroxylase similar to that found in *Melilotus officinalis* (Gestetner and Conn 1974) but not yet identified in tobacco. In this context, it is worth mentioning that a rapid biosynthesis of a coumarin scopoletine and its glucoside scopolin was observed in leaves of tobacco cultivar Samsun elicited with glycoprotein isolated from culture medium of *Phytophtora megasperma* (Costet et al. 2002) or with a sulphonated oligoglucan, laminarin (Ménard et al. 2004). Because it is believed that the early biosynthetic steps in scopoletine biosynthesis involve hydroxylation in the *ortho* position of an appropriate C_6–C_3 precursor, such a modification of *trans*-cinnamic acid might also take place. However, in tobacco cultivar Xanthi no scopoletine or scopolin were detected before or after elicitation (Costet et al. 2002). This suggests that in the Xanthi cultivar some of the biosynthetic steps leading to scopoletine formation are inactive.

Acknowledgements – We wish to thank Prof. Aleksandra Skłodowska and Dr Karol Bal from the Laboratory of Environmental Pollution Analysis, Faculty of Biology, Warsaw University, for the HPLC and GCMS analyses. This research

was in part financially supported by grant 2 P04A 073 26 from the State Committee for Scientific Research (Poland).

References

Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature 406: 512–515

Arimura G, Ozawa R, Horiuchi J, Nishioka T, Takabayashi J (2001) Plant–plant interactions mediated by volatiles emitted from plants infested by spider mites. Biochem Syst Ecol 29: 1049–1061

Baldwin IT, Schultz JC (1983) Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants. Science 221: 277–279

Bell EA, Charlwood BV (1980) Secondary Plant Products. Encyclopedia of Plant Physiology, New Series 8. Springer-Verlag, Berlin, Heidelberg, New York

Bestwick CS, Brown IR, Mansfield JW (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiol 118: 1067–1078

Bjorklund JA, Leete E (1992) Biosynthesis of the benzoyl moiety of cocaine from cinnamic acid via (R)-(+)-3-hydroxy-3-phenylpropanoic acid. Phytochemistry 31: 3883–3887

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Boatright J, Negre F, Chen X, Kish CM, Wood B, Peel G, Orlova I, Gang D, Rhodes D, Dudareva N (2004) Understanding in vivo benzenoid metabolism in petunia petal tissue. Plant Physiol 135: 1993–2011

Chadha KC, Brown SA (1974) Biosynthesis of phenolic acids in tomato plants infected with *Agrobacterium tumefaciens*. Can J Bot 52: 2041–2046

Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E (2003) An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. Plant J 36: 577–588

Chong J, Pierrel MA, Atanassova R, Werck-Reichhart D, Fritig B, Saindrenan P (2001) Free and conjugated benzoic acid in tobacco plants and cell cultures. Induced accumulation upon elicitation of defense responses and role as salicylic acid precursors. Plant Physiol 125: 318–328

Chu A, Furlan M, Davin LB, Zajicek J, Towers GHN, Soucy-Breau CM, Rettig SJ, Croteau R, Lewis NG (1994) Phenylbutanoid and taxane-like metabolites from needles of *Taxus brevifolia*. Phytochemistry 36: 975–985

Cleland CF, Ajami A (1974) Identification of the flower-inducing factor isolated from aphid Honeydew as being salicylic acid. Plant Physiol 54, 904–906

Costet L, Fritig B, Kauffmann S (2002) Scopoletin expression in elicitor-treated and tobacco mosaic virus-infected tobacco plants. Physiol Plant 115(2): 228–235

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rela M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. Science 266: 1247–1250

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, Bonham C, Woodc C (2000) Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant Cell 12: 949–962

El-Basyouni S, Chen D, Ibrahim RK, Neish AC, Towers GHN (1964) The biosynthesis of hydroxybenzoic acids in higher plants. Phytochemistry 3: 485–492

El-Mawla AMA, Beerhues L (2002) Benzoic acid biosynthesis in cell cultures of *Hypericum androsaemum*. Planta 214: 727–733

Engelbert J, Alborn HT, Schmelz EA, Tumlinson JH (2004) Airborne signals prime plants against insect herbivore attack. Proc Natl Acad Sci USA 101: 1781–1785

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc Natl Acad Sci USA 89: 2480–2484

Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA 87: 7713–7716

French CJ, Vance CP, Towers GHN (1976) Conversion of *p*-coumaric acid to *p*-hydroxybenzoic acid by cell free extracts of potato tubers and *Polyporus hispidus*. Phytochemistry 15: 564–566

Funk C, Brodelius PE (1990) Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. III. Conversion of 4-methoxycinnamic acids into 4-hydroxybenzoic acids. Plant Physiol 94: 102–108

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754–756

Gestetner B, Conn EE (1974) The 2-hydroxylation of *trans*-cinnamic acid by chloroplasts from *Melilotus alba* Desr. Arch Biochem Biophys 163: 617–624

Jarvis AP, Schaaf O, Oldham NJ (2000) 3-Hydroxy-3-phenylpropanoic acid is an intermediate in the biosynthesis of benzoic acid and salicylic acid but benzaldehyde is not. Planta 212: 119–126

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science 291: 2141–2144

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lawton K, Weymann K, Friedrich L, Vernooij B, Uknes S, Ryals J (1995) Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol Plant Microbe Interact 8: 863–870

Lee H-I, Leon J, Raskin I (1995) Biosynthesis and metabolism of salicylic acid. Proc Natl Acad Sci USA 92: 4076–4079

León J, Yalpani N, Raskin I, Lawton MA (1993) Induction of benzoic acid 2-hydroxylase in virus-inoculated tobacco. Plant Physiol 103: 323–328

Lösher R, Heide L (1994) Biosynthesis of *p*-hydroxybenzoate from *p*-coumarate and *p*-coumaroyl-coenzyme A in cell-free extracts of *Lithospermum erytrorhizon*, cell cultures. Plant Physiol 106: 271–279

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science 250: 1002–1004

Martínez C, Pons E, Prats G, León J (2004) Salicylic acid regulates flowering time and links defense responses and reproductive development. Plant J 37: 209–217

Mehlhorn H, Lelandais M, Korth HG, Foyer CH (1996) Ascorbate is the natural substrate for plant peroxidases. FEBS Lett 378: 203–206

Ménard R, Alban S, de Ruffray P, Jamois F, Franz G, Fritig B, Yvin JC, Kauffmann S (2004) Beta-1, 3 glucan sulfate, but not beta-1, 3 glucan, induces the salicylic acid signaling pathway in tobacco and *Arabidopsis*. Plant Cell 16: 3020–3032

Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250: 1004–1006

Op den Camp R, Kuhlemeier C (1997) Aldehyde dehydrogenase in tobacco pollen. Plant Mol Biol 35: 355–365

Podstolski A, Havkin-Frenkel D, Malinowski J, Blount JW, Kourteva G, Dixon RA (2002) Unusual properties of 4-hydroxybenzaldehyde synthase activity from tissue cultures of the vanilla orchid *Vanilla planifolia*. Phytochemistry 61: 611–620

Van Poecke RM, Dicke M (2002) Induced parasitoid attraction by *Arabidopsis thaliana*: involvement of the octadecanoid and the salicylic acid pathway. J Exp Bot 53: 1793–1799

Van Poecke RM, Posthumus MA, Dicke M (2001) Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. J Chem Ecol 27: 1911–1928

Rasmussen JB, Smith JA, Williams S, Burkhart W, Ward E, Somerville SC, Ryals J, Hammerschmidt R (1995) cDNA cloning and systemic expression of acidic peroxidases associated with systemic acquired resistance to disease in cucumber. Physiol Mol Plant Pathol 46: 389–400

Ribnicky DM, Shulaev V, Raskin I (1998) Intermediates of Salicylic acid biosynthesis in tobacco. Plant Physiol 118: 565–572

Ross, AF (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology 14: 340–358

Ryals J, Lawton KA, Delaney TP, Friedrich L, Kessmann H, Neuenschwander U, Uknes S, Vernooij B, Weymann K (1995) Signal transduction in systemic acquired resistance. Proc Natl Acad Sci USA 92: 4202–4205

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner H-Y, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819

Schnitzler JP, Madlung J, Rose A, Seitz HU (1992) Biosynthesis of *p*-hydroxybenzoic acid in elicitor-treated carrot cell cultures. Planta 188: 594–600

Shulaev V, Silverman P, Raskin I (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. Nature 385: 718

Venooij B, Friedrich L, Morse ARR, Kolditz-Jawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell 6: 959–965

Walker TS, Bais HP, Halligan KM, Stermitz FR, Vivanco JM (2003) Metabolic profiling of root exudates of *Arabidopsis thaliana*. J Agric Food Chem 51: 2548–2554

White RF (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology 99: 410–412

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature 414: 562–565

Wu H, Haig T, Pratley J, Lemerle D, An M (2002) Biochemical basis for wheat seedling allelopathy on the suppression of annual ryegrass (*Lolium rigidum*). J Agric Food Chem 50: 4567–4571

Yalpani N, Silverman P, Wilson TM, Kleier DA, Raskin I (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 3: 809–818

Yalpani N, León J, Lawton MA Raskin I (1993) Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol 103: 315–321

Yazaki K, Heide L, Tabata M (1991) Formation of *p*-hydroxybenzoic acid from *p*-coumaric acid by cell free extracts of *Lithospermum erytrorhizon* cell cultures. Phytochemistry 30: 2233–2236

Zenk MH (1965) Biosynthese von Vanilin in *Vanilla planifolia* Andr. Z Pflanzenphysiol 53: 404–414

Edited by A. Crozier