Free and Conjugated Benzoic Acid in Tobacco Plants and Cell Cultures. Induced Accumulation upon Elicitation of Defense Responses and Role as Salicylic Acid Precursors[1]

Julie Chong[2], Marie-Agnès Pierrel[2], Rossitza Atanassova[3], Danièle Werck-Reichhart, Bernard Fritig, and Patrick Saindrenan*

Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université Louis Pasteur, 67084 Strasbourg cedex, France (J.C., M.-A.P., R.A., B.F., P.S.); and Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université Louis Pasteur, 67083 Strasbourg cedex, France (D.W.-R.)

Salicylic acid (SA) is a key endogenous component of local and systemic disease resistance in plants. In this study, we investigated the role of benzoic acid (BA) as precursor of SA biosynthesis in tobacco (*Nicotiana tabacum* cv Samsun NN) plants undergoing a hypersensitive response following infection with tobacco mosaic virus or in tobacco cell suspensions elicited with β-megaspermin, an elicitor from *Phytophthora megasperma*. We found a small pool of conjugated BA in healthy leaves and untreated cell suspensions of tobacco, whereas free BA levels were barely detectable. Infection of plants with tobacco mosaic virus or elicitation of cells led to a rapid de novo synthesis and accumulation of conjugated BA, whereas free BA was weakly induced. In presence of diphenylene iodonium, an inhibitor of superoxide anion formation, SA accumulation was abolished in elicited cells and much higher BA levels were concomitantly induced, mainly as a conjugated form. Furthermore, piperonylic acid, an inhibitor of cinnamate-4-hydroxylase was used as a powerful tool to redirect the metabolic flow from the main phenylpropanoid pathway into the SA biosynthetic branch. Under these conditions, in vivo labeling and radioisotope dilution experiments with [^{14}C]trans-cinnamic acid as precursor clearly indicated that the free form of BA produced in elicited tobacco cells is not the major precursor of SA biosynthesis. The main conjugated form of BA accumulating after elicitation of tobacco cells was identified for the first time as benzoyl-glucose. Our data point to the likely role of conjugated forms of BA in SA biosynthesis.

Salicylic acid (SA, 2-hydroxybenzoic acid) is considered one of the key endogenous signals involved in the activation of numerous plant defense responses (Shah and Klessig, 1999). Early evidence showed that application of SA induced resistance against several pathogens and the expression of pathogenesis-related proteins in a variety of plants (White, 1979; Ward et al., 1991). These properties subsequently were found to mimic the natural defense response in tobacco (*Nicotiana tabacum*) and cucumber when elevated endogenous SA levels were correlated with induced resistance to the invading pathogen (Malamy et al., 1990; Métraux et al., 1990). Further studies have implicated SA as an essential component in the induction of systemic acquired resistance (SAR) in several plant species (Shah and Klessig, 1999). SAR is a pathogen-inducible broad spectrum resistance phenomenon occurring in tissues distant from the primary infection. The most compelling evidence of the key role of SA in both local and systemic disease resistance came from transgenic tobacco and Arabidopsis overexpressing a bacterial salicylate hydroxylase that catalyzes the conversion of SA to inactive catechol. Infected transgenic plants are unable to express SAR and also show reduced local resistance to pathogens (Gaffney et al., 1993; Delaney et al., 1994). However, although essential in tissue expressing SAR, SA itself is probably not the translocated signal responsible for activating defense responses in the uninoculated parts of the plant (Vernooij et al., 1994). Later on, several genetic screens identified Arabidopsis mutants that are impaired in SA perception or accumulation and exhibit an enhanced susceptibility to pathogen infection (Cao et al., 1994; Delaney et al., 1995; Nawrath and Métraux, 1999).

The genetic manipulation of the rate of SA synthesis in plants should provide a way to engineer high levels of pathogen resistance. However, although significant progress has been made recently to establish the role of SA in disease resistance, the genes encoding critical enzymes of SA biosynthesis as well as the rate-limiting step in SA production are still largely unknown. A large body of evidence suggests that SA

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[2] These authors contributed equally to the paper.

[3] Present address: ERS CNRS 6099, Batiment Botanique, 40, avenue du Recteur Pineau, 86022 Poitiers cedex, France.

* Corresponding author; e-mail patrick.saindrenan@ibmp-ulp.u-strasbg.fr; fax 33–3–88–61–44–42.

derives from the shikimate-phenylpropanoid pathway (Zenk and Müller, 1964). In this pathway, Phe is first converted to trans-cinnamic acid (*t*-CA) by the Phe ammonia-lyase enzyme (PAL), *t*-CA is then either hydroxylated to *o*-coumaric acid before oxidation of the side chain, or the side chain of *t*-CA is shortened to benzoic acid (BA), which in turn is hydroxylated at the ortho position (Sticher et al., 1997). At least in tobacco and in cucumber, the biosynthetic pathway of SA was proposed to proceed exclusively via free BA (Yalpani et al., 1993; Meuwly et al., 1995). Hydroxylation of BA at the C-2 position would be catalyzed by a soluble Cyt P450 monooxygenase, benzoate-2-hydroxylase (BA2H), as reported in tobacco (Leon et al., 1995b). The side chain of *t*-CA could be shortened by a nonoxidative mechanism, which was described for the biosynthesis of *p*-hydroxybenzoic acid in several systems and involves the formation of *p*-hydroxybenzaldehyde as intermediate (French et al., 1976; Schnitzler et al., 1992). However, the shortening of the side chain of C6-C3 compounds is generally believed to proceed oxidatively by a mechanism similar to the β-oxidation of fatty acids. Biochemical evidence for a β-oxidation pathway was provided in *Lithospermum erythrorhizon* where the biosynthesis of *p*-hydroxybenzoic acid proceeds via *p*-coumaroyl-coenzyme A (CoA), which is oxidized and cleaved to *p*-hydroxybenzoyl-CoA and acetyl-CoA in a thioclastic reaction (Löscher and Heide, 1994). Besides CoA thioesters, phenylpropanoid Glc esters might also serve as activated intermediates in the side chain shortening reactions (Funk and Brodelius, 1990).

Intermediates of SA biosynthesis have been previously studied in tobacco where the induced HR to tobacco mosaic virus (TMV) was artificially extended to the whole plant by a temperature shift procedure, allowing a massive induction of SA biosynthesis. Under these conditions, SA accumulation was suggested to result from the release of free BA from a large preformed pool of conjugated BA (Yalpani et al., 1993); this free BA in turn would induce BA2H activity converting BA to SA (Leon et al., 1995a). In this sequence, the accumulation of high SA levels does not necessarily require an increase in PAL activity. However, activation of the phenylpropanoid pathway was shown to be a prerequisite for the biosynthesis of SA in cucumber (Meuwly et al., 1995) and Arabidopsis (Mauch-Mani and Slusarenko, 1996). If BA per se appears to play a central role in SA biosynthesis, until now the exact form of BA acting as an intermediate remains elusive (Ribnicky et al., 1998).

We have developed a specific method for BA determination and show in this report that conjugated BA is not present as a large preformed pool in tobacco cv Samsun NN. We also investigated the kinetics of accumulation of free and conjugated BA and SA during the HR of tobacco to TMV as well as in elicited Bright Yellow (BY) tobacco cells. Furthermore, in vivo labeling experiments conducted in this study clearly indicate that the free form of BA is not the major intermediate of SA biosynthesis in elicited tobacco cells.

RESULTS

Time Course of BA and SA Accumulation in TMV-Infected Tobacco Leaves and in Elicited Tobacco Cells

Levels of free and conjugated BA and SA were determined both during the HR of tobacco plants to TMV and in BY tobacco cells treated with β-megaspermin, an HR-inducing proteinaceous fungal elicitor. In contrast to previous data describing a large constitutive pool of conjugated BA (100 $\mu g\ g^{-1}$ fresh weight) in untreated leaves of tobacco cv Xanthi-nc (Yalpani et al., 1993), untreated tobacco leaves of the cv Samsun NN contained only 4 $\mu g\ g^{-1}$ fresh weight of conjugated BA, whereas the levels of free BA were low (0.2 $\mu g\ g^{-1}$ fresh weight; Fig. 1A). Moreover, in our hands, the levels of conjugated BA in healthy cv Xanthi-nc leaves were in the same range (5 $\mu g\ g^{-1}$ fresh weight). Inoculation of cv Samsun NN with TMV triggered a strong accumulation of conjugated

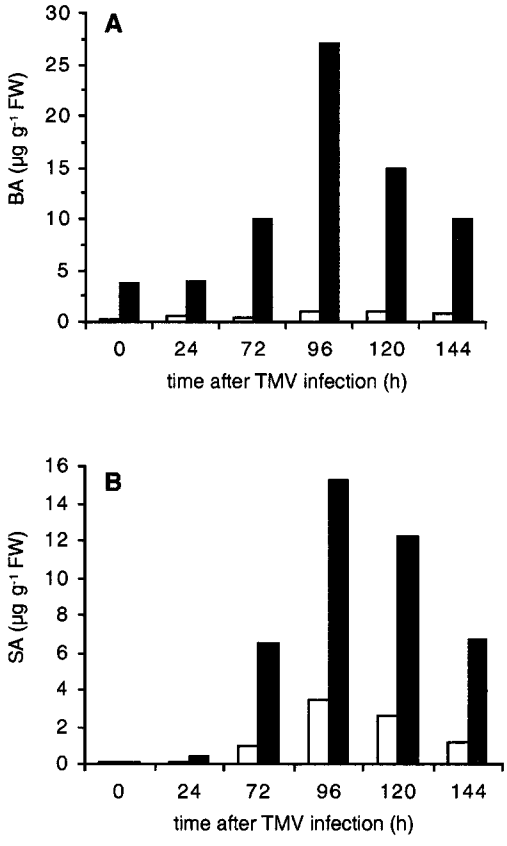

Figure 1. Levels of BA (A) and SA (B) after TMV infection. Free (white bars) and conjugated (black bars) BA and SA were determined in tobacco cv Samsun NN leaves after infection with TMV.

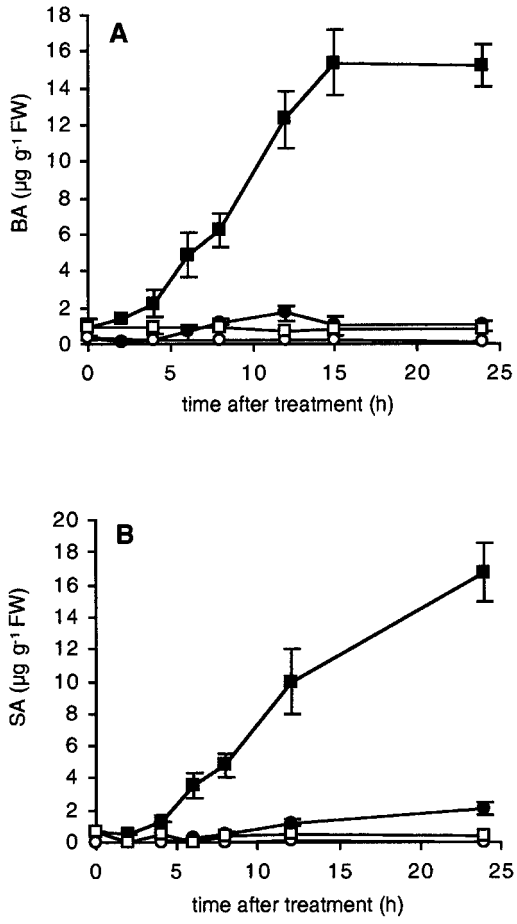

Figure 2. Levels of BA (A) and SA (B) in elicited tobacco cells. Free (●, ○) and conjugated (■, □) BA and SA were determined in BY tobacco cells treated with 50 nM β-megaspermin (●, ■) or water (○, □). Results are means ± SD of three independent experiments.

BA, peaking after 96 h, and decreasing thereafter (Fig. 1A). Free BA was only weakly induced after TMV infection and appeared as a minor component of the BA pool (Fig. 1A). TMV inoculation also induced an increase in free and conjugated SA, which began 3 d after infection and paralleled BA accumulation (Fig. 1B).

Similar results were obtained with elicited tobacco cells (Fig. 2). Conjugated BA began to increase 4 h after elicitation and was maximal after 15 h, with levels 20 times higher than in H_2O-treated cells (Fig. 2A). Elicitor treatment also induced a slight increase in free BA, but the free form was approximately 10 times less abundant than conjugated BA. Kinetics of free and conjugated SA also paralleled those of BA in elicited cells (Fig. 2B).

These results show that in tobacco, BA occurs mainly as the conjugated form and that both free and conjugated BA accumulated during the HR to TMV and after elicitation of cell suspension cultures with β-megaspermin.

Inhibition of Elicitor-Induced SA Accumulation by Diphenylene Iodonium Parallels an Increase in BA Accumulation in Tobacco Cells

It has been previously shown that the inhibition of active oxygen species production by diphenylene iodonium (DPI) completely abolished SA accumulation induced by β-megaspermin treatment of tobacco cells (Dorey et al., 1999). Since BA is thought to be a direct precursor of SA in several plants, the fate of BA in elicited cells in the presence of DPI was analyzed.

Whereas treatment of BY tobacco cells with β-megaspermin induced a strong SA accumulation, simultaneous DPI application completely abolished the elicitor-induced SA production (Fig. 3A). The results observed for BA levels were strikingly different since BY tobacco cells elicited in the presence of DPI accumulated 2 to 3 times more free BA and 6 to 7 times more conjugated BA than cells elicited in the absence of DPI (Fig. 3B).

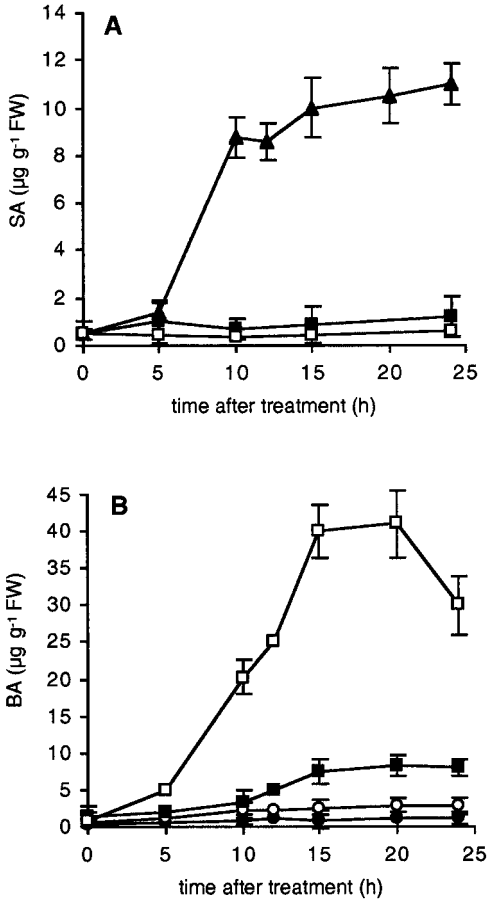

Figure 3. Effect of DPI on SA (A) and BA (B) accumulation in elicited tobacco cells. A, Levels of total SA in cells treated with 50 nM β-megaspermin (▲), 50 nM β-megaspermin + 5 μM DPI (■) or DPI alone (□). B, Levels of free (●, ○) and conjugated (■, □) BA in BY cells treated with 50 nM β-megaspermin (●, ■) or 50 nM β-megaspermin + 5 μM DPI (○, □). Results are means ± SD of three independent experiments.

These results show a clear correlation between the inhibition by DPI of SA accumulation after elicitation and the concomitant increase in BA accumulation, mainly as conjugated BA. Therefore, BA appears as a likely intermediate in SA biosynthesis in planta. If free BA is the direct precursor of SA, the strong accumulation of conjugated BA in elicited cells treated with DPI may arise from the rapid conjugation of the free form, which is no longer converted to SA. Alternatively, conjugated forms of BA could also represent direct intermediates in SA biosynthesis.

Is Free BA the Key Intermediate in SA Biosynthesis?

To establish whether free BA is the major intermediate in SA biosynthesis, we conducted in vivo radiolabeling experiments with [^{14}C]*t*-CA in BY-2 elicited tobacco cells. BY-2 tobacco cells are well suited to in vivo labeling experiments because they are highly homogenous with respect to cell type and do not aggregate to form cell clusters. Exogenously applied precursors such as [^{14}C]Phe or [^{14}C]*t*-CA have been used in several studies for determination of intermediates in SA biosynthesis, but most of the labeling was usually incorporated into lignin and phytoalexin precursors instead of being directed to the SA branch (Meuwly et al., 1995). To increase the labeling of SA biosynthetic intermediates, we used piperonylic acid, a natural molecule that behaves as a potent inactivator of cinnamate-4-hydroxylase (Schalk et al., 1998).

Treatment of tobacco BY-2 cells with exogenous unlabeled *t*-CA (100 μM) did not lead to an increase in SA accumulation (0.15 $\pm$ 0.01 μg g^{-1} fresh weight) compared with untreated cells (0.12 $\pm$ 0.01 μg g^{-1} fresh weight). However, simultaneous application of 100 μM *t*-CA and 10 μM piperonylic acid induced a strong accumulation of SA (3.2 $\pm$ 0.12 μg g^{-1} fresh weight), which was 30 times higher than in cells treated with *t*-CA alone. Moreover, levels of SA were slightly increased in tobacco cells treated with piperonylic acid alone (0.25 $\pm$ 0.02 μg g^{-1} fresh weight) compared with control cells (0.12 $\pm$ 0.01 μg g^{-1} fresh weight). This indicates that most of both exogenously applied and endogenous *t*-CA are likely to be metabolized by the main phenylpropanoid pathway downstream of *para*-coumaric acid (*p*-CO) in the absence of piperonylic acid. Together, our data show that piperonylic acid effectively inhibits the metabolic flow to C6-C3 compounds, allowing a diversion of the flow of metabolites to C6-C1 compounds such as SA.

For in vivo radiolabeling experiments, BY-2 tobacco cells were treated with β-megaspermin in presence or in absence of 10 μM piperonylic acid for 11 h and then fed [^{14}C]*t*-CA for 1 h. Total radioactivity associated with *t*-CA, *p*-CO, BA, and SA was first measured in hydrolyzed methanolic extracts. Figure 4 shows that radioactivity incorporated into BA and SA was low compared with the radioactivity detected in *p*-CO, confirming that the flow of metabolites is mainly directed to the general phenylpropanoid pathway, to the detriment of SA biosynthesis both in elicited and in control cells. Treatment of elicited tobacco cells with piperonylic acid abolished the incorporation of radioactivity in *p*-CO with a concomitant retention in *t*-CA (Fig. 4). Under these conditions, the flow of precursor (*t*-CA) was redistributed and the radioactivity associated to BA and SA was strongly enhanced (Fig. 4). Piperonylic acid thus appears as a powerful tool for increasing the radiolabeling of the intermediates in the SA pathway. The use of piperonylic acid enabled us to measure and compare the specific radioactivities of free BA and free SA.

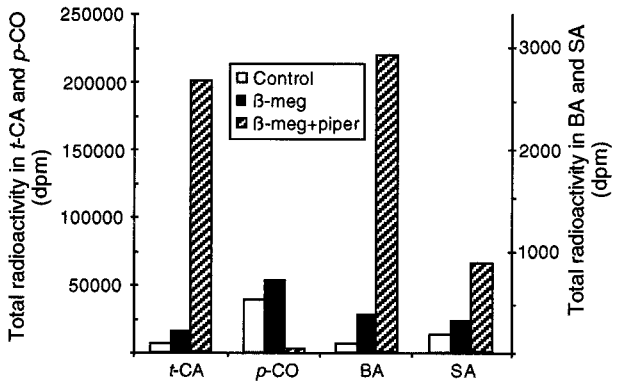

Figure 4. Effect of piperonylic acid on the distribution of total radioactivity (dpm) associated with total *t*-CA, *p*-CO, BA, and SA after feeding [^{14}C]*t*-CA to elicited tobacco cells. BY-2 cells were elicited with 50 nM β-megaspermin for 12 h in presence (hatched bars) or in absence (black bars) of 10 μM piperonylic acid. Control cells (white bars) were treated with water. [^{14}C]*t*-CA (2.2 μmol, 4.5 mCi mmol^{-1}) was added during the last hour of elicitation. Total radioactivity associated with *t*-CA, *p*-CO, BA, and SA was determined after acid and base hydrolysis of the extracts. Note the difference of the scale used for *t*-CA and *p*-CO, and BA and SA. Piper, Piperonylic acid; β-meg, β-megaspermin. A duplicate experiment gave essentially the same results.

BY-2 tobacco cells were elicited in presence of 10 μM piperonylic acid with or without exogenous unlabeled BA (100 μM) for 12 h. Pulse labeling was conducted with [^{14}C]*t*-CA during the last hour of elicitation. As expected, the addition of exogenous free BA led to an increase in the endogenous pool of free BA (Fig. 5A). However, levels of free SA were not much enhanced by free BA addition (Fig. 5A).

If free BA is a direct precursor of SA, the addition of unlabeled BA should result in a reduction of the specific radioactivity associated to SA. In the presence of exogenous unlabeled BA, the specific radioactivity of free BA was very significantly reduced but unexpectedly, the specific radioactivity of free SA was not lowered (Fig. 5B). When tobacco cells were elicited in presence of exogenous BA, the specific radioactivity of free BA was actually 10-fold lower compared with that of free SA (Fig. 5B). However, these results do not exclude conjugated forms of BA

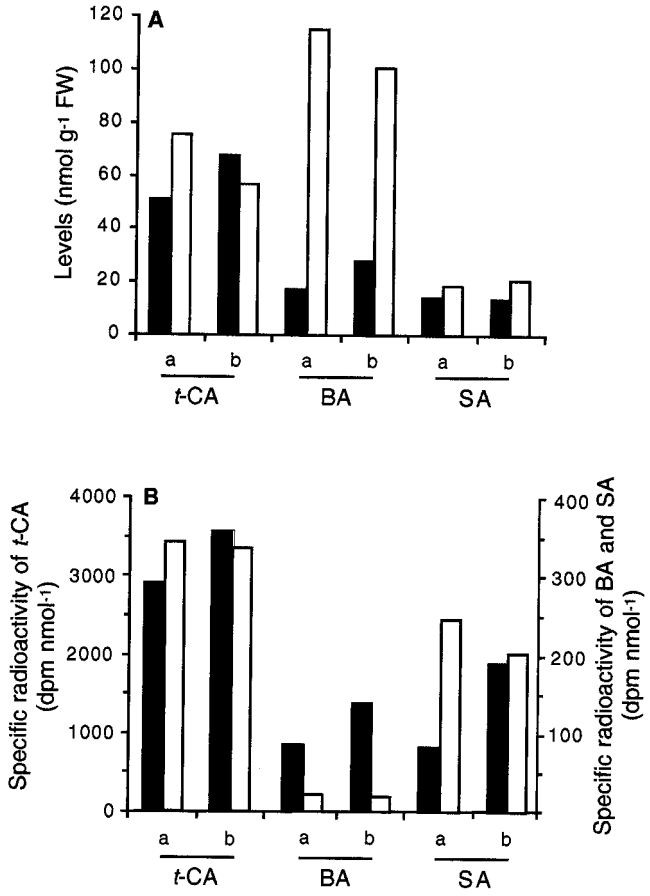

Figure 5. Effect of the addition of unlabeled BA on the levels of free *t*-CA, BA, and SA (A) and on the specific radioactivities associated to the free forms of *t*-CA, BA, and SA (B) after labeling with [^{14}C]*t*-CA. BY-2 tobacco cells were treated simultaneously for 12 h with 50 nM β-megaspermin and 10 μM piperonylic acid in presence (white bars) or in absence (black bars) of 100 μM unlabeled BA. [^{14}C]*t*-CA (2.2 μmol, 4.5 mCi mmol^{-1}) was added during the last hour of elicitation. a and b correspond to the results of two independent experiments.

as SA precursors. The specific activity of conjugated BA was determined in elicited cells in presence of piperonylic acid and [^{14}C]*t*-CA and appeared to be in the same range (155 dpm nmol^{-1}) in presence or in absence of exogenous unlabeled BA.

Identification of Conjugated BA as Benzoyl-Glc in Elicited BY Tobacco Cells

The strong accumulation of conjugated BA in elicited cells prompted us to examine more closely the nature of this conjugate. The presence of BA conjugate(s) was first established by measuring an increase in the amount of free BA after saponification of an extract of elicited tobacco cells or TMV-infected tobacco leaves with 1 N NaOH at room temperature. Treatment of these extracts with β-glucosidase from almond released the same amount of free BA as base hydrolysis, indicating that the BA conjugate is probably benzoyl-Glc (data not shown). To further identify the conjugate, BY tobacco cells were elicited in presence of 10 μM piperonylic acid for 11 h and then fed [^{14}C]*t*-CA for 1 h. Thin-layer chromatography (TLC) analysis of a methanolic extract of these cells showed a strong incorporation of radioactivity into a compound comigrating with an authentic standard of benzoyl-Glc. The compound comigrating with benzoyl-Glc (R_F = 0.7) was eluted and further purified by C18 reversed-phase HPLC, where it co-eluted again with chemically-synthesized benzoyl-Glc (retention time = 22.8 min; Fig. 6A). UV spectrum of the compound purified from tobacco cells (peak 1) and of reference benzoyl-Glc were identical with λmax, 233.8 nm, and 275.1 nm (Fig. 6A). The compound corresponding to the HPLC peak 1 was also subjected to hydrolysis with trifluoroacetic acid. After hydrolysis, the sugar moiety was identified as Glc by TLC (data not shown). Furthermore, the chemical struc-

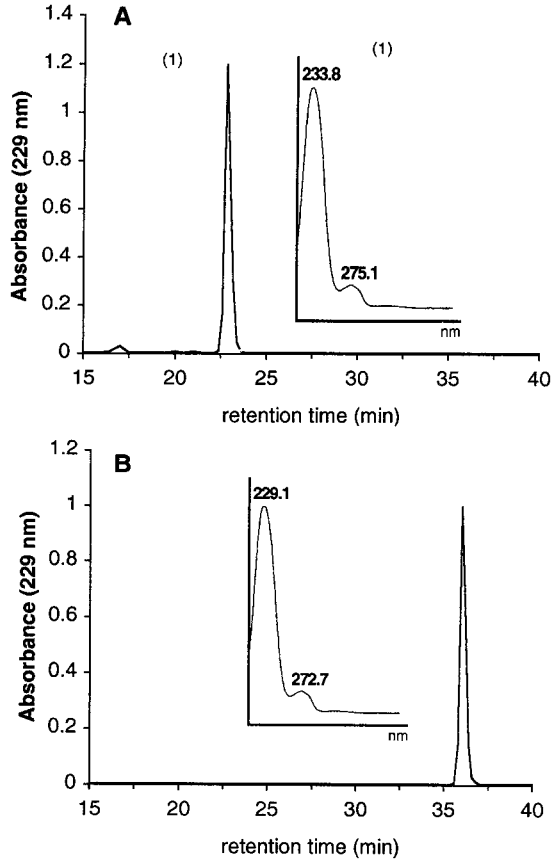

Figure 6. Identification of benzoyl-Glc in elicited BY tobacco cells. Cell cultures were treated simultaneously with 50 nM β-megaspermin and 10 μM piperonylic acid for 12 h. [^{14}C]*t*-CA (2.2 μmol, 4.5 mCi mmol^{-1}) was added during the last hour of elicitation. Methanolic extract of elicited cells was separated by TLC and further purified by HPLC (see "Material and Methods"). A, HPLC profile of the benzoyl-Glc fraction separated by TLC. Peak 1 was identified as benzoyl-Glc on the basis of its retention time and its UV spectrum. B, HPLC profile of the peak 1 after hydrolysis with β-glucosidase from almond. The aglycon was identified as free BA on the basis of its retention time and its UV spectrum.

ture of the BA conjugate was confirmed by hydrolysis with β-glucosidase of the compound eluting at 22.8 min. After β-glucosidase digestion, the peak at 22.8 min disappeared, and a new peak at 36 min became apparent (Fig. 6B). The retention time of this product was identical to that of free BA as demonstrated by cochromatography with authentic BA. The product released by β-glucosidase also displayed the same UV spectrum as free BA with λmax, 229.1 nm, and 272.7 nm (Fig. 6B).

Together, these results demonstrate that BA is esterified to Glc and that benzoyl-Glc is the major BA conjugate in elicited tobacco cells because all the BA conjugate pool could be hydrolyzed by β-glucosidase, which specifically releases β-linked terminal D-Glc.

DISCUSSION

Elevated levels of conjugated BA (100 $\mu g\ g^{-1}$ fresh weight) have been reported to occur in healthy leaves of tobacco cv Xanthi-nc and the depletion of this large BA conjugate pool observed after TMV inoculation of tobacco plants incubated at 32°C, and then shifted to 24°C was shown to be correlated with a rapid increase in free BA and SA levels (Yalpani et al., 1993). This observation has led to the proposal that SA accumulation does not necessarily require de novo BA synthesis from *t*-CA and that free BA is the immediate precursor of SA biosynthesis (Yalpani et al., 1993; Leon et al., 1995a). Our aim was to investigate the precise role of BA as a precursor of SA biosynthesis in tobacco undergoing HR to TMV and in tobacco cell suspension cultures elicited with β-megaspermin, a system mimicking the natural HR in plants (Baillieul et al., 1996).

In the present study, we detected the presence of a rather small pre-existing pool of conjugated BA in healthy tobacco with levels approximately 20-fold lower than those previously reported (Yalpani et al., 1993). However, in cucumber and potato the BA pool was below the limit of detection even after pathogen infection (Meuwly et al., 1995; Coquoz et al., 1998). We can offer no clear explanation to such discrepancies in the determination of BA levels in healthy tobacco leaves, but the sole cinnamic acid derivative known to be naturally in the range of 100 $\mu g\ g^{-1}$ fresh weight in tobacco is 5-caffeoyl quinic acid (Fritig et al., 1972; Tanguy and Martin, 1972). It is known that levels of BA in plant materials are difficult to determine accurately and unambiguously (Coquoz et al., 1998). Moreover, we found that free BA was steam distillable and was lost when reducing the samples to dryness in vacuo (see "Material and Methods"). We have developed a specific separation method that resolves BA from all other components of the plant extracts. This method implies first a fractionation by TLC allowing the separation of BA from co-eluting cinnamic acid derivatives in tobacco extracts and second, an HPLC analysis using sensitive UV detection. With this procedure, we were able to monitor for the first time the kinetics of accumulation of free and conjugated BA (a) in tobacco expressing the HR under natural conditions and (b) after treatment of cell cultures with an elicitor of defense responses (Figs. 1A and 2A). In both model systems, a massive build-up of conjugated forms of BA was induced, while free BA was scarcely increased, reflecting either a rapid conversion of the free form to conjugated forms or its low release from the induced bound forms. Kinetics of SA accumulation paralleled those of BA in the two model systems. In contrast to our results, dramatic increase in free BA was reported in TMV-inoculated tobacco leaves incubated at 32°C and then shifted to 24°C (Yalpani et al., 1993). This difference may reflect the use of different experimental conditions, i.e. temperature shift versus natural conditions of HR induction. BA accumulation was also more sustained in β-megaspermin-treated cells than in TMV-infected leaves, where conjugated BA levels tend to decrease after 4 d. This difference may result from a more pronounced extent of cell death in infected leaves compared with elicited cells.

In vivo labeling studies with exogenously applied precursors have implicated free BA as the direct precursor of SA in tobacco and cucumber. Dorey et al. (1999) showed that application of DPI to elicitor-treated tobacco cells prevented SA accumulation. Here, we demonstrate that the inhibition of SA production after elicitation in presence of DPI was strictly correlated with the accumulation of higher BA levels, mainly as conjugated BA. Such loss of function experiments strongly support a role for endogenous BA as SA precursor in planta. Moreover, the mechanism by which DPI inhibits elicitor-induced SA accumulation in tobacco cells has to be determined. DPI is a suicide inhibitor of the mammalian NADPH oxidase (O'Donnell et al., 1993) producing superoxide anion that is subsequently dismutated to water. Our results demonstrate that active oxygen species produced after elicitation are necessary for the conversion of BA to SA. In support of this is the fact that exogenous hydrogen peroxide, which induced the accumulation of SA in tobacco, has been proposed to activate SA biosynthesis by stimulating the BA2H activity converting BA to SA (Leon et al., 1995a). Another possibility is that the conversion of BA to SA occurs via the iron-driven Fenton reaction producing hydroxyl radicals that can attack aromatic molecules such as BA to generate hydroxylated compounds (Gutteridge, 1987).

To assess the exact role of free BA in SA biosynthesis, we conducted in vivo radiolabeling experiments using [^{14}C]*t*-CA as precursor in elicited BY-2 tobacco cells. Previous studies have used labeled *t*-CA or Phe as precursors to elucidate SA biosynthesis. However, feeding Phe or *t*-CA to uninoculated tobacco did not result in SA accumulation (Yalpani et

al., 1993). It is possible that exogenous *t*-CA is rapidly metabolized by esterification with Glc as in elicitor-treated *Phaseolus vulgaris* cells (Edwards et al., 1990). In TMV-infected tobacco seedlings or infected cucumber plants, no radioactivity was found in the putative SA biosynthetic intermediates after feeding [^{14}C]*t*-CA or [^{14}C]Phe (Yalpani et al., 1993; Meuwly et al., 1995). In cucumber, addition of unlabeled *p*-coumarate was found necessary to drive high amounts of [^{14}C]Phe toward the synthesis of SA rather than to the lignin and phytoalexin precursors (Meuwly et al., 1995).

We have previously shown that piperonylic acid, a specific in vitro inhibitor of cinnamate-4-hydroxylase, decreases the formation of *p*-CO in elicited tobacco cells and the accumulation of the 7-hydroxylated coumarin scopoletin in TMV-infected tobacco leaves (Schalk et al., 1998). In the present study, treatment of tobacco cells with piperonylic acid alone triggered a small but significant increase of SA levels (0.25 ± 0.02 versus 0.12 ± 0.01 $\mu g\ g^{-1}$ fresh weight in control cells), showing that the metabolic flow in the SA pathway was very low in nonelicited cells. In contrast, simultaneous addition of piperonylic acid and *t*-CA resulted in a strong accumulation of SA compared with cells treated with *t*-CA alone. This demonstrates that the availability of *t*-CA, rather than the conversion of BA to SA, is limiting for SA formation. This assumption is reinforced by the fact that exogenous BA triggers the formation of SA, whereas *t*-CA does not (Yalpani et al., 1993; Pierrel, Atanassova, and Saindrenan, unpublished data). Rasmussen and Dixon (1999) have also shown that the addition of *t*-CA to PAL-overexpressing cells resulted in higher levels of hydroxycinnamic acid derivatives with no modification of the SA pool. Piperonylic acid thus reveals the importance of the distribution of the metabolic flow between the C6-C3 and the C6-C1 pathways and provides the first tool available for redirecting the main metabolic flow toward the synthesis of SA.

Labeling experiments in the presence of piperonylic acid clearly demonstrated the conversion of *t*-CA to BA and SA in elicited tobacco cells. However, the specific activity of free BA was much lower compared with that of free SA in the presence of exogenous unlabeled free BA (Fig. 5). This result does not follow the basic principles of radioisotope labeling of intermediates in a biosynthetic pathway, which predicts that the specific radioactivity of a precursor is higher than the specific radioactivity of its product, because of the dilution of the isotope within each intermediate pool, provided that the initial radioactive precursor is fed by pulse labeling. On the contrary, our data show that the conversion of [^{14}C]*t*-CA to SA is not affected by the addition of unlabeled BA. Recent experiments using [^{13}C]Phe in temperature-shift experiments with TMV-inoculated tobacco leaf discs have also shown that SA was to some extent more labeled than BA (Ribnicky et al., 1998). Hence, it appears that in elicited tobacco cells, the free form of BA is probably not the key intermediate in SA biosynthesis. However, other studies showed that feeding BA to untreated tobacco resulted in an efficient conversion to SA (Yalpani et al., 1993). Therefore, our results suggest that in elicited tobacco cells, SA is mainly produced from an alternative pathway that does not involve free BA.

A plausible explanation is that the metabolic pathway to SA proceeds through conjugated form(s) of BA. This hypothesis is reinforced by the fact that the specific activity of conjugated BA was in the same range as the specific activity of free SA and was unaffected by the addition of free BA.

The most likely hypothesis is that SA is synthesized via the formation of CoA thioesters, in a mechanism analogous to the β-oxidation of fatty acids. In *L. erythrorhizon*, the enzymatic formation of *p*-hydroxybenzoic acid from *p*-CO in cell-free extracts is thought to proceed exclusively via *p*-coumaroyl-CoA (Löscher and Heide, 1994). SA biosynthesis via CoA thioesters would first involve the formation of cinnamoyl-CoA. It is interesting that the conversion of cinnamoyl-CoA to benzoyl-CoA has already been shown to occur in mammals (Mao et al., 1994). In addition, benzoyl-CoA is an intermediate in the synthesis of dianthramide phytoalexins in elicited cells of *Dianthus caryophillus* (Reinhard and Matern, 1989). This benzoyl-CoA thioester could represent in tobacco the immediate precursor of salicyloyl-CoA, which in turn could be converted to SA by thioesterases (Fig. 7).

We have identified the bulk of the pool of esterified BA as benzoyl-Glc in elicited cells, but additional BA conjugates with a rapid turnover and which do not accumulate may also exist in tobacco. The glucosylation of BA may be regarded as a detoxication reaction producing a less reactive form of storage. However, we cannot rule out the possibility that Glc esters of cinnamic acid derivatives are intermediates in SA biosynthesis, since earlier investigations with *Vanilla planifolia* cell cultures showed that the formation of vanillic acid proceeded via the formation of Glc esters of cinnamic acids (Funk and Brodelius, 1990). Moreover, we have recently characterized a tobacco glucosyltransferase rapidly induced during the HR to TMV and acting on phenylpropanoid derivatives, particularly on *t*-CA (Fraissinet-Tachet et al., 1998). Tanaka and Kojima (1991) also reported the characterization of an enzyme from *Ipomoea batatas* hydroxylating p-coumaroyl-Glc at the C-3 position. This makes possible that salicyloyl-Glc ester previously identified in tobacco (Edwards, 1994) is synthesized directly from benzoyl-Glc (Fig. 7).

In conclusion, our data show that SA accumulation requires de novo BA synthesis from *t*-CA and are in accordance with previous studies reporting that treatment with PAL inhibitors reduced the accumu-

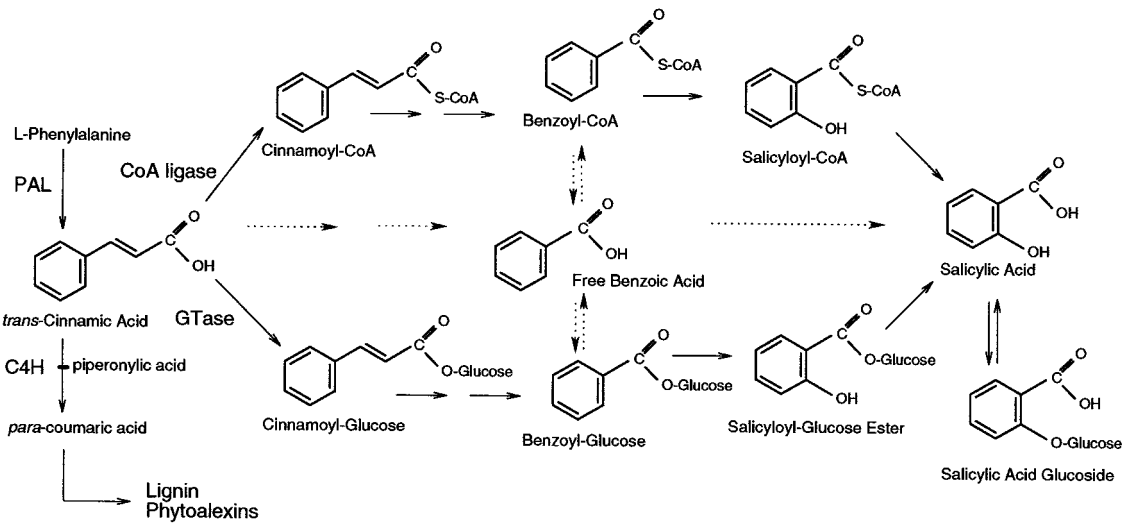

Figure 7. Proposed pathways of SA biosynthesis in tobacco involving CoA thioesters or Glc esters. Radioisotope dilution experiments indicate that free BA is not the major intermediate of SA biosynthesis. The metabolic pathway to SA may therefore proceed via conjugated forms of BA. In the branch involving Glc esters, salicyloyl-Glc ester might represent a biosynthetic intermediate, whereas SA glucoside would be a storage form. GTase: glucosyltransferase (UDP-Glc:cinnamic acid glucosyltransferase).

lation of SA in infected tobacco (Dorey et al., 1997), Arabidopsis (Mauch-Mani and Slusarenko, 1996), and cucumber (Meuwly et al., 1995). However, free BA is probably not the major intermediate in SA biosynthesis, which may involve conjugated forms of BA. Future studies will be directed at elucidating which BA conjugates, i.e. CoA thioester or Glc ester represent activated intermediates in SA biosynthesis.

MATERIALS AND METHODS

Biological Materials and Treatments

Tobacco (*Nicotiana tabacum* cv Samsun NN) was maintained in growth chambers at 25°C with a 16-h photoperiod. For TMV infection, 4-week-old tobacco plants were inoculated by rubbing fully expanded leaves with a virus suspension (0.2 μg mL^{-1}). At different times after treatment, leaf tissue was harvested, quickly frozen in liquid nitrogen, and stored at −80°C until analysis.

BY-cultured tobacco cells (derived from *N. tabacum* cv Bright Yellow) were a gift of Professor Clarence Ryan (Institute of Biological Chemistry, Washington State University, Pullmann). Cells were grown at pH 5.8 in Murashige and Skoog medium (Fraissinet-Tachet et al., 1998). Six-day-old BY cells were induced with 50 nM β-megaspermin, a proteinaceous HR-inducing elicitor isolated from *Phytophthora megasperma* (Baillieul et al., 1996). For inhibition experiments, 5 μM DPI was added 5 min before treatment with β-megaspermin.

For in vivo labeling experiments, the homogenous, synchronized tobacco BY-2 cell suspension was used instead of BY cells. BY-2 cells were maintained as described by Nagata et al. (1992). Synchronization of the cells was achieved according to Reichheld et al. (1995) with 3 mg mL^{-1} aphidicolin and 1.5 mg mL^{-1} propyzamide. Elicitor treatment of cell suspension cultures was performed 48 h after aphidicolin treatment with 50 nM β-megaspermin. Piperonylic acid (10-μM final concentration) was added at the same time as β-megaspermin. Eleven hours after elicitation in presence or in absence of piperonylic acid, BY-2 tobacco cells (20-mL batches) were incubated with 2.2 μmol of [^{14}C]*t*-CA (4.5 mCi mmol^{-1}). After 1 h, the cells were washed with Murashige and Skoog medium, harvested, and frozen in liquid nitrogen. For radioisotope dilution experiments, unlabeled BA (100-μM final concentration) was added at the same time as β-megaspermin.

Chemicals

BA, *t*-CA, *p*-CO, DPI, and piperonylic acid were from Sigma-Aldrich (St. Quentin Fallavier, France). Benzoyl-Glc was a generous gift from Dr. Philippe Desbordes (Aventis, Lyon, France) and was synthesized as described by Klick and Herrmann (1988). [7-^{14}C]BA was obtained from NEN (Zaventem, Belgium). [3-^{14}C]*t*-CA was from Isotopchim (Ganagobie-Peyruis, France). β-megaspermin was kindly provided by Dr. Serge Kauffmann (Institut de Biologie Moléculaire des Plantes-Centre National de la Recherche Scientifique, Strasbourg, France).

Extraction and HPLC Analysis of *t*-CA, *p*-CO, BA, and SA

Plant material (2 g) was extracted with 4 mL of 90% (v/v) MeOH. After centrifugation, the residue was extracted again with 100% (v/v) MeOH. The combined extracts were reduced to dryness under nitrogen instead of in vacuo because free BA was found to be steam distillable. Indeed, the evaporation in vacuo to dryness of a 90% (v/v) methanolic solution of radiolabeled BA showed that 95% of

the radioisotope was recovered in the distillate. [^{14}C]BA (10 nCi, 17.5 mCi mmol^{-1}) was added to each sample before extraction as internal standard for correction of losses. For each sample, the dried extract was resuspended in 1 mL of water at 50°C. For free BA analysis, samples were acidified to 1 N HCl and extracted twice with 2 volumes of ether. For determination of conjugated BA, extracts were saponified with 1 N NaOH for 30 min at room temperature. After neutralization and acidification, samples were extracted with ether and used for total BA determination. Ether phases were dried under nitrogen. Conjugated BA content was assigned as the difference between total and free BA. Extracts were redissolved in 200 μL of ethyl acetate, and BA was first separated by TLC on 0.25-mm silicagel plates developed in toluene:acetic acid:water (6:7:3, v/v/v, upper phase). Compounds comigrating with an authentic standard of BA (R_F = 0.62) were eluted twice with 90% (v/v) MeOH at 50°C. The methanolic solution was evaporated under nitrogen before HPLC analysis. Radioactivity was determined by liquid scintillation counting of an aliquot sample.

Total *t*-CA and *p*-CO were extracted according to the same protocol except that the extract was submitted to both alkaline and acid (2 N HCl final concentration, at 80°C during 40 min) hydrolyses. Free and total SA were extracted as described by Baillieul et al. (1995).

HPLC analysis of SA, BA, *t*-CA, and *p*-CO was performed on a 5-μm C18 Nova Pak column (150 × 4 mm, Waters, St. Quentin-en-Yvelines, France), using a gradient of CH_3CN in 25 mM NaH_2PO_4, pH 3, at a flow rate of 1 mL min^{-1}. The gradient was 5% to 22% for 35 min and then 22% to 80% for 1 min. BA, *t*-CA, and *p*-CO were detected by UV spectrophotometry at 229, 290, and 280 nm, respectively. SA was detected by fluorescence (λ_{ex} = 315 nm, λ_{em} = 405 nm). Identification of the compounds was based on co-chromatography with authentic standards coupled to a photodiode array detector (maxplot between 230 and 400 nm, Waters Millenium software). Compounds were quantified by comparison with reference standards. For radiolabeling experiments, fractions of 200 μL were collected and counted in a liquid scintillation counter.

Identification of Benzoyl-Glc in BY Tobacco Cells

BY tobacco cells were simultaneously treated with 50 nM β-megaspermin and 10 μM piperonylic acid for 11 h and then fed [^{14}C]*t*-CA for 1 h. Cells (20 g) were harvested by vacuum filtration and extracted twice with 90% (v/v) MeOH. Aqueous MeOH was evaporated under reduced pressure at 30°C. The dried residue was resuspended in 100% (v/v) MeOH and analyzed by TLC on 0.25-mm silicagel plates developed in *n*-butanol:acetic acid:water (4:1:1, v/v/v). Radioactivity on TLC plates was visualized with a Bio-Imager Analyzer (Fuji, Tokyo). Radioactive compounds comigrating with an authentic standard of benzoyl-Glc (R_F = 0.7) were eluted from silicagel with 90% (v/v) MeOH at 50°C. After evaporation, compounds were redissolved in 250 μL of 5% (v/v) acetonitrile in 25 mM NaH_2PO_4, pH 3. One aliquot was applied on a 5-μm C18 Uptisphere HPLC column (150 × 4 mm, Interchrom, Montluçon, France) and eluted in 25 mM NaH_2PO_4, pH 3, with an increasing CH_3CN gradient (0–5 min, 5% [v/v] CH_3CN; 5–40 min, 5%–22% [v/v] CH_3CN; 41–46 min, 80% [v/v] CH_3CN at a flow rate of 1 mL min^{-1}). Benzoyl-Glc was detected by UV spectrophotometry at 229 nm. One aliquot of the HPLC peak corresponding to the compound co-eluting with reference benzoyl-Glc was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 100°C. The resulting sugars were separated by TLC on 0.1-mm cellulose plates developed in *n*-butanol:acetic acid:water (3:1:1, v/v/v) followed by ethyl acetate:pyridine:water (10:4:3, v/v/v). Detection of sugar spots with aniline hydrogen-phtalate was performed as described by Franke et al. (1998). Another aliquot of the HPLC peak was incubated for 2 h at 37°C with 1 unit of β-glucosidase from almond (Sigma-Aldrich) in 50 mM sodium acetate buffer, pH 5.5, and analyzed by HPLC as described above.

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