Induction of UDP-Glucose:Salicylic Acid Glucosyltransferase Activity in Tobacco Mosaic Virus-Inoculated Tobacco (Nicotiana tabacum) Leaves

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Salicylic acid (SA) is a putative signal that activates plant resistance to pathogens. SA levels increase systemically following the hypersensitive response produced by tobacco mosaic virus (TMV) inoculation of tobacco (Nicotiana tabacum L. cv Xanthi-nc) leaves. The SA increase in the inoculated leaf coincided with the appearance of a β-glucosidase-hydrolyzable SA conjugate identified as β-O-d-glucosylsalicylic acid (GSA). SA and GSA accumulation in the TMV-inoculated leaf paralleled the increase in the activity of a UDP-glucosylsalicylic acid 3-O-glucosyltransferase (EC 2.4.1.35) (β-GTase) capable of converting SA to GSA. Healthy tissues had constitutive β-GTase activity of 0.076 milliunits g⁻¹ fresh weight. This activity started to increase 48 h after TMV inoculation, reaching its maximum (6.7-fold induction over the basal levels) 72 h after TMV inoculation. No significant GSA or elevated β-GTase activity could be detected in the healthy leaf immediately above the TMV-inoculated leaf. The effect of TMV inoculation on the β-GTase and GSA accumulation could be duplicated by infiltrating tobacco leaf discs with SA at the levels naturally produced in TMV-inoculated leaves (2.7–27.0 μg g⁻¹ fresh weight). Pretreatment of leaf discs with the protein synthesis inhibitor cycloheximide inhibited the induction of β-GTase by SA and prevented the formation of GSA. Of 12 analogs of SA tested, only 2,6-dihydroxybenzoic acid induced β-GTase activity.

During SAR, pathogen-free plant tissues become more resistant to subsequent attack by a wide range of pathogens (Ross, 1961a, 1961b). Following TMV inoculation, SA levels in Xanthi-nc tobacco increase systemically (Malamy et al., 1990; Enyedi et al., 1992). The first increase in the level of SA occurs 36 h after inoculation, coincidentally with the appearance of HR (Malamy et al., 1990). The highest levels of SA are found in and around TMV-induced necrotic lesions (Enyedi et al., 1992).

Tobacco leaves rapidly metabolize exogenously supplied or endogenously produced SA to GSA (Enyedi et al., 1992). Therefore, most of the SA in TMV-inoculated leaves of tobacco is present in the form of GSA (Enyedi et al., 1992; Malamy et al., 1992). GSA was found only in leaves that exhibited HR. Phloem sap and pathogen-free leaves of TMV-inoculated tobacco did not contain significant levels of GSA (Enyedi et al., 1992).

Various hydroxybenzoic acid glucosides have been reported to occur in higher plants (Griffiths, 1959; Ibrahim and Towers, 1959; Klambt, 1962; Umetani et al., 1990). Hypocotyls of sunflower incubated with carbox-y-labeled benzoic acid formed trace amounts of SA and larger amounts of GSA (Klambt, 1962). SA-inducible glucosyltransferases, which catalyze the glucosylation of SA to SA-glucosides, have been partially purified from cell-suspension cultures of Mallotus japonicus (Tanaka et al., 1990) and from oat roots (Yalpani et al., 1992b).

This paper describes how the local and systemic induction of tobacco β-GTase during HR to TMV relates to the temporal and spatial distribution of SA and GSA. It demonstrates that (a) β-GTase activity increases following TMV inoculation; (b) the increase in β-GTase activity coincides with the accumulation of GSA; (c) the SA levels present in the inoculated leaves are sufficient for the induction of β-GTase activity; (d) systemic increases in SA are not sufficient for the induction of β-GTase activity, which may explain the lack of significant amounts of GSA in the uninoculated leaves; and (e) β-GTase induction is specific for SA.

Abbreviations: CHX, cycloheximide; GSA, β-O-d-glucosylsalicylic acid; β-GTase, UDP-glucosylsalicylic acid 3-O-glucosyltransferase (EC 2.4.1.35); HR, hypersensitive response; SA, salicylic acid; SAR, systemic acquired resistance; TMV, tobacco mosaic virus.
MATERIALS AND METHODS

Plant Material

All experiments were performed with leaves of tobacco (Nicotiana tabacum L. cv Xanthi-nc NN genotype) grown as described previously (Enyedi et al., 1992). The uppermost, near-fully expanded leaves of 6- to 8-week-old plants were dusted with dry carborundum (240 grit) and inoculated by gently rubbing the upper leaf surface with 200 μL of a suspension of U1 strain TMV (25 μg mL⁻¹ in 0.1 m potassium phosphate buffer, pH 6.7). Leaves were rinsed with deionized water immediately following inoculation. Control plants were dusted with carborundum and mock inoculated with 200 μL of 0.1 m potassium phosphate buffer (pH 6.7). Following inoculation, plants were maintained at 24°C under continuous illumination provided by cool-white fluorescent lamps (200 pmol m⁻² s⁻¹).

For SA infiltration experiments, 22-mm-diameter leaf discs were infiltrated through the abaxial surface with SA solutions ranging in concentration from 0 to 1 mM using a 1-cc syringe. Following infiltration, leaf discs were kept on wet filter paper inside 14-cm Petri dishes at 24°C under cool-white fluorescent lamps (200 μmol m⁻² s⁻¹). Leaf discs were harvested at various times, frozen in liquid nitrogen, and stored at -80°C for future determinations of β-GTase activity and SA and GSA levels.

Extraction and Analysis of β-GTase Activity

β-GTase was extracted from tobacco leaf tissue using a modified method of Yalpani et al. (1992b). Frozen tissue (700 mg) was pulverized to a fine powder in liquid nitrogen using mortar and pestle and transferred to a chilled 15-mL Corex tube. One milliliter of extraction buffer (25 mM Tris-Mes, 10% [v/v] glycerol, 20 mM β-mercaptoethanol, 1 μM leupeptin, pH 6.5) containing 1% (w/w leaf) polyvinylpolypyrrolidone was added to the frozen tissue and the contents of the tube were allowed to thaw on ice. The resulting slurry was vigorously mixed and then centrifuged at 10,500g for 20 min at 4°C to pellet any remaining leaf tissue. For β-GTase activity, the standard assay mixture (200 μL final volume) contained 16 μL of assay buffer (25 mM Tris-Mes, 0.4 mM SA, 1.2 kBq [7-¹⁴C]SA [2.1 GBq mmol⁻¹], 10 mM UDP-Glc, pH 6.5). The reaction was started by adding 184 μL of crude extract (15,500 g supernatant) to the assay buffer and briefly mixing the sample. The reaction was carried out at 30°C and terminated after 30 min with the addition of 200 μL of methanol. To separate [¹⁴C]GSA from [¹⁴C]SA, the reaction mixture was passed through a Polyamide-6 (poly-caprolactam/Perlon/Nylon-6, Serva Biochemicals, Paramus, NJ) ion-exchange column (10-mL volume) equilibrated in 10 mM Tris-Mes (pH 6.5) (Yalpani et al., 1992a). [¹⁴C]GSA was eluted with 5 mL of deionized H₂O and radioactivity was determined with the Bio-Rad protein assay using BSA fraction V (1 mg mL⁻¹) as the standard.

Extraction and Quantitation of SA

SA was extracted from tobacco leaf tissue (0.5-g sample) and quantified by HPLC as described (Enyedi et al., 1992). All data were corrected for SA recovery, which ranged from 44 to 52%.

Hydrolysis of Leaf Extracts

To determine the GSA content, methanolic leaf extract was dried and resuspended in 500 μL of hydrolysis buffer (100 mM sodium acetate buffer, pH 5.5) containing 20 units of β-glucosidase (EC 3.2.1.21; almond). After 1.5 h of incubation at 37°C, extracts were acidified to pH 1.0 with TCA acid and subjected to SA extraction and quantitation (Enyedi et al., 1992).

CHX Treatment

Leaf discs were infiltrated with CHX (40 μg mL⁻¹) and allowed to incubate for 30 min. Control discs were infiltrated with deionized H₂O. Following incubation, leaf discs were infiltrated with 1 mM SA (35 μg g⁻¹ fresh weight) or deionized H₂O, incubated for an additional 6 h, harvested, and assayed for β-GTase activity, GSA, and SA as described above.

Effects of SA Analogs

The induction of β-GTase activity in tobacco leaf discs was assessed using 12 structural analogs of SA (see Fig. 5). Discs were infiltrated with 20 μL of a 1.0 mM solution of each test compound as described for SA. β-GTase was extracted from frozen discs and its activity assayed as described above. To test for possible interference of SA analogs with the β-GTase assay, standard enzyme assays were performed in which each analog was added to a final concentration of 0.4 mM.

Experimental Design and Statistical Analysis

All experiments were performed with a minimum of three tissue sample replicates per treatment or sampling time. Each experiment was repeated at least twice with similar results. Data from each experiment are expressed as the mean ± se, and paired t tests were performed to determine significant differences between treatments at the P ≤ 0.05 level (Steel and Torrie, 1980).

RESULTS

Induction of β-GTase Activity in TMV-Inoculated Leaves

The time course of the induction of β-GTase activity in TMV-inoculated tobacco leaves was established and related to the tissue levels of free and total SA (SA plus GSA) (Fig. 1). Basal levels of β-GTase activity (approximately 0.08 milliunits g⁻¹ fresh weight or 0.01 milliunits mg⁻¹ of protein) were present at the time of inoculation. The highest levels of β-GTase activity were observed 72 h after TMV inoculation, when β-GTase activity in the infected leaves increased 6.7-
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Figure 1. β-GTase activity expressed as milliunits (mU) g⁻¹ fresh weight (A) and mU mg⁻¹ of protein (B), and total SA (SA plus GSA) content (C) in TMV-inoculated leaves of Xanthi-nc tobacco at various times after inoculation. Vertical bars denote ± se. An asterisk (*) denotes a significant level of β-GTase induction at the P ≤ 0.05 level. GSA content is derived by subtraction of B from C; for example, GSA is 67% of the total at 84 h after inoculation.

Figure 2. β-GTase activity expressed as milliunits (mU) mg⁻¹ of protein (A) and mU g⁻¹ fresh weight (B), and free and total SA (SA plus GSA) content (C) in the leaf immediately above the TMV-inoculated leaf at various times after inoculation. Vertical bars denote ± se.

Exogenous SA induced β-GTase activity in tobacco leaf discs in a dose-dependent manner (Fig. 3A). No significant induction was observed in leaf discs exposed to SA below 0.27 μg g⁻¹ fresh weight. At 2.7 μg g⁻¹ fresh weight, SA produced the first significant increase in β-GTase activity. This dose corresponded to the lower range of SA levels naturally produced in the TMV-inoculated leaf (Fig. 1B). Increasing the SA dose to 13.5 and 27.0 μg g⁻¹ fresh weight

time course of transcriptional activation of PR-1 genes in TMV-inoculated tobacco leaves (Malamy et al., 1990).

No increase in β-GTase activity was observed in the leaf immediately above the TMV-inoculated leaf (Fig. 2, A and B). Reflecting the constitutively low β-GTase activity, uninoculated leaves did not accumulate GSA (Fig. 2C). However, the levels of free SA in the uninoculated leaves increased from 50 to 175 ng g⁻¹ fresh weight 72 h after TMV inoculation of the lower leaf (Fig. 2C). The fact that for some time points the level of total SA was less than the level of free SA may be explained by experimental error.

Induction of β-GTase Activity by SA

Exogenous SA induced β-GTase activity in tobacco leaf discs in a dose-dependent manner (Fig. 3A). No significant induction was observed in leaf discs exposed to SA below 0.27 μg g⁻¹ fresh weight. At 2.7 μg g⁻¹ fresh weight, SA produced the first significant increase in β-GTase activity. This dose corresponded to the lower range of SA levels naturally produced in the TMV-inoculated leaf (Fig. 1B). Increasing the SA dose to 13.5 and 27.0 μg g⁻¹ fresh weight

fold over the basal levels when expressed on a milliunit g⁻¹ fresh weight basis (Fig. 1A). The first significant increase in β-GTase activity was observed 48 h after inoculation. This increase corresponded to the appearance of necrotic lesions and an increase in the level of both free and conjugated SA (Fig. 1, B and C). Twenty-four and 36 h after inoculation, leaf tissues contained only basal levels of SA (approximately 75 ng g⁻¹ fresh weight). Also, little if any SA conjugate was present in the leaves for 36 h after TMV inoculation. Paralleling the increase in β-GTase activity, SA and GSA levels in the inoculated leaves began to rise 48 h after inoculation. Eighty-four hours after TMV inoculation, leaves contained 26.8 μg g⁻¹ fresh weight of total SA, 67% of which was present in the form of GSA. It is interesting that the time course of β-GTase induction paralleled the earlier established

Figure 3. β-GTase activity expressed as [m] milliunits (mU) g⁻¹ fresh weight and (A) mU mg⁻¹ of protein (A), free SA content (B), and total SA (SA plus GSA) content (C) in Xanthi-nc tobacco leaf discs infiltrated with various amounts of SA and incubated for 6 h. Vertical bars denote ± se. An asterisk (*) denotes a significant level of β-GTase induction at the P ≤ 0.05 level.

caused a proportionally greater induction of β-GTase activity.

Figure 4. Effect of CHX on β-GTase activity expressed as milliunits (mU) mg⁻¹ of protein (A) and mU g⁻¹ fresh weight (B), and free and total SA content (C) in Xanthi-nc tobacco leaf discs treated with SA. Leaf discs were infiltrated with SA (35 μg g⁻¹ fresh weight) 30 min after CHX treatment and incubated for an additional 6 h. Vertical bars denote ± se.

Induction of β-GTase Activity by SA Analogs

To test the specificity of β-GTase induction by SA, leaf discs were infiltrated with a 1-mM solution of 12 different analogs of SA (Fig. 5). Only 2,6-dihydroxybenzoic acid induced β-GTase activity to a level comparable with that induced with SA. Benzoic and α-coumaric acid also increased

inhibitor CHX (Fig. 4, A and B). When leaf discs were pretreated with CHX and infiltrated with 1 mM SA, the level of extractable β-GTase activity did not significantly differ from those in leaf discs treated with H₂O or CHX alone (Fig. 4, A and B). The ability of CHX to inhibit β-GTase induction was reflected by the greater level of free SA and by the absence of GSA 6 h after SA infiltration (Fig. 4C). As expected, control leaf discs not exposed to CHX incorporated most of the infiltrated SA into GSA (Fig. 4C).

CHX Effects

The induction of β-GTase activity was effectively blocked by pretreatment of leaf discs with the protein synthesis inhibitor CHX (Fig. 4, A and B). When leaf discs were pretreated with CHX and infiltrated with 1 mM SA, the level of extractable β-GTase activity did not significantly differ from those in leaf discs treated with H₂O or CHX alone (Fig. 4, A and B). The ability of CHX to inhibit β-GTase induction was reflected by the greater level of free SA and by the absence of GSA 6 h after SA infiltration (Fig. 4C). As expected, control leaf discs not exposed to CHX incorporated most of the infiltrated SA into GSA (Fig. 4C).
The physiological role of GSA in TMV-inoculated leaves is unknown. The SA-to-GSA conversion may lead to sequestration of the SA glucoside into the vacuole (Ben-Tal and Cleland, 1982). The ability of SA to induce β-GTase activity may serve as an effective mechanism for the feedback regulation of SA levels in plant tissues. This regulation of tissue SA may be an important control point in the signal transduction pathway leading to the activation of disease-resistance mechanisms in plants. Alternatively, GSA may function as a storage pool for SA.

Our data demonstrate that β-GTase is one of the many proteins induced during the HR response. Therefore, it is
appropriate to refer to this enzyme as a pathogenesis-related protein. However, the particular importance of this protein is its ability to regulate the levels of SA—a putative plant hormone and a signal molecule in pathogen-resistance responses (Raskin, 1992). Future studies of this enzyme could lead to a better understanding of plant-pathogen interactions.

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LITERATURE CITED


Holmes FO (1938) Inheritance of resistance to tobacco mosaic disease in tobacco. Phytopathology 28: 553–561

Ibrahim RK, Towers GHN (1959) Conversion of salicylic acid to gentisic acid and o-pyrocatechic acid, all labelled with carbon-14, in plants. Nature 184: 1803


