Downregulation of a Pathogen-Responsive Tobacco UDP-Glc:Phenylpropanoid Glucosyltransferase Reduces Scopoletin Glucoside Accumulation, Enhances Oxidative Stress, and Weakens Virus Resistance

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INTRODUCTION

Plants are characterized by their ability to synthesize numerous different secondary metabolites, among them phenylpropanoids, which are derived from Phe and fulfill a wide range of important biological functions (Dixon and Paiva, 1995). It is well established that phenylpropanoid metabolism is one of the major metabolic pathways stimulated during the hypersensitive response (HR), a very efficient mechanism of induced disease resistance in plants. The HR is characterized by localized cell and tissue death at the site of infection and is associated with the induction of intense metabolic alterations, resulting in confinement of the pathogen (Hammond-Kosack and Jones, 1996; Fritig et al., 1998). One of the earliest responses underlying HR cell death in plants is the increase in the production of reactive oxygen intermediates (ROIs), giving rise to the so-called oxidative burst (Hammond-Kosack and Jones, 1996).

Among the ROIs, O2·− and H2O2 may be key mediators of cell death characterizing the HR (Dangl et al., 1996). On the other hand, H2O2 from the oxidative burst also could act as a diffusible signal for the induction of protectant genes in cells adjacent to HR lesions, thereby limiting oxidant-mediated cell death (Lamb and Dixon, 1997). The cells surrounding the HR lesion actually are stimulated strongly without being destined to die, and they produce a large set of defense responses that contribute to the efficient restriction of pathogen spread (Dorey et al., 1997; Fritig et al., 1998). In tobacco treated with an HR-inducing elicitor, this narrow zone of living cells expressing sustained defense responses is characterized by blue fluorescence under UV light, which is attributable to the accumulation of phenylpropanoids (Dorey et al., 1997).

Many of the pathogen-induced phenylpropanoids (e.g., coumarins and isoflavonoids) are considered phytoalexins because they exhibit antimicrobial properties in vitro and accumulate to antimicrobial concentrations in plant tissues...
upon infection (Dixon and Paiva, 1995; Kuc, 1995; He and Dixon, 2000). Besides their antimicrobial properties, phenylpropanoids such as the ubiquitous chlorogenic acid (3-cafeoylquinic acid) display antioxidant properties in vitro (Rice-Evans et al., 1997), and hydroxycoumarins such as scopoletin (6-methoxy-7-hydroxycoumarin) have been proposed to act in planta as scavengers of ROI excess produced after triggering of the HR (Chong et al., 1999). Moreover, salicylic acid (SA) originating from the phenylpropanoid pathway (Coquoz et al., 1998; Chong et al., 2001) plays key roles in the signaling network leading to the establishment of both local and systemic resistance (Gaffney et al., 1993; Delaney et al., 1994).

Phenylpropanoids rarely accumulate in their free form in plant cells but often are conjugated to sugars, most often Glc, through the action of UDP-Glc:glucosyltransferases (UGTs) (Vogt and Jones, 2000). Many properties of the aglycones are regulated by glucosylation. Secondary metabolite glucosides generally exhibit reduced chemical reactivity and enhanced water solubility and can act as storage and transport forms (Li et al., 2001). Glucosylation thus plays a general role in the accumulation of phenylpropanoids that would be toxic in their free form; in addition, phenylpropanoid glucosides also are believed to be of importance in plant defense. The 4-O-β-D-glucosides of monolignols are considered to serve as transport forms of lignin monomers into the cell wall, leading to enhanced lignification after pathogen attack (Whetten et al., 1998). Glc esters of hydroxycinnamic acids also are thought to act as intermediates for trans-esterification and impregnation of cinnamate derivatives into the cell wall (Hahlbrock and Scheel, 1989). Moreover, conjugation reactions catalyzed by UGTs may be critical in regulating the levels of signaling compounds such as SA by converting active molecules to inactive conjugated forms (Hennig et al., 1993).

Despite the remarkable capacity of plants to glucosylate a wide range of different chemical structures, UGTs were largely unknown until recently. Analysis of the completed Arabidopsis genome indicates the existence of >100 genes encoding putative glucosyltransferases, but the functions of most of them have not been elucidated (Li et al., 2001). However, within the last 2 years, cloning and substrate specificity analysis of several plant phenylpropanoid UGTs acting on anthocyanidins, flavonoids, and hydroxycinnamic acids have shed new light on the functions of some members of the UGT superfamily (Ford et al., 1998; Vogt et al., 1999; Hirotani et al., 2000; Vogt and Jones, 2000; Lim et al., 2001).

Among the UGTs that could be involved in plant defense, a SA- and pathogen-inducible UGT acting in vitro on the signaling molecules SA, benzoic acid, and cinnamic acid has been isolated in tobacco (Lee and Raskin, 1999). Recently, we showed that two tobacco genes (Togt1 and Togt2), which are induced early by SA and during an HR (Horvath and Chua, 1996; Fraissinet-Tachet et al., 1998), encode UGTs that act efficiently on hydroxycoumarins, especially scopoletin (Figure 1), and on hydroxycinnamic acids (Fraissinet-Tachet et al., 1998). These genes are homologous with a glucosyltransferase gene from tomato (Twi1) that responds rapidly to wound- and pathogen-related signals (O’Donnell et al., 1998).

One way of identifying unambiguously the role of UGTs in planta is to manipulate their expression. Until now, the isolation of the maize bronze-1 transposon-tagging mutants was the only example of the unequivocal identification of the function of a flavonol-3-O-glucosyltransferase (Fedoroff et al., 1984). The modulation of a specific UGT in vivo and the study of its effect on phenylpropanoid metabolism and defense mechanisms have not been performed. Using a loss-of-function approach based on tobacco salicylic acid– and pathogen-inducible UGT (TOGT) antisense inhibition in transgenic plants, we show clearly the function of TOGT in hydroxycoumarin glucosylation and the crucial role of this UGT in virus resistance. Our work further assigns new roles for scopoletin in plant defense, as both an antiviral compound and an antioxidant molecule involved in the regulation of ROI accumulation.

RESULTS

Generation of Transgenic Tobacco Plants with Reduced TOGT Levels

We previously showed that TOGT is encoded by two genes, Togt1 and Togt2, in tobacco (Fraissinet-Tachet et al., 1998).

![Figure 1. Biosynthetic Pathway to Scopoletin and Scopolin.](image)

Scopoletin synthesis involves the first catalytic steps of the phenylpropanoid pathway, leading to p-coumaric acid. Ferulic acid has been demonstrated as a scopoletin precursor in cultured tobacco cells (Fritig et al., 1970); however, specific enzymes of scopoletin biosynthesis are not known. In vitro, scopoletin is glucosylated very efficiently by TOGT (Fraissinet-Tachet et al., 1998). C4H, cinnamate-4-hydroxylase.
Both genes are expressed at similar levels upon elicitation of defense responses or treatment with SA, and they share 95% nucleotide identity in the coding region. To downregulate the expression of both Togt genes, the complete coding sequence of Togt1 was introduced in the antisense orientation downstream from the constitutive 35S promoter of Cauliflower mosaic virus into tobacco. Togt antisense expression in plants had no effect on normal growth and development.

Because Togt gene expression is known to be induced rapidly by SA (Horvath and Chua, 1996; Fraissinet-Tachet et al., 1998), antisense inhibition was evaluated after SA treatment of plantlets. Among the T1 progeny, two lines, AS 14 and AS 25, showed a considerable reduction in TOGT protein levels after treatment with SA (Figure 2A). The segregation analysis further indicated that these lines were single-locus insertions (data not shown). The inhibition was particularly evident in plants of the AS 14 line, in which TOGT was almost undetectable. The amount of residual TOGT protein was measured by quantifying the 52-kD TOGT bands revealed in protein gel blot experiments (Figure 2B). After treatment with SA, when the mean TOGT protein level of plants transformed with the empty vector was set to 100%, the mean TOGT protein levels of the AS 14 and AS 25 lines were 2 and 25%, respectively (Figure 2B). These results indicate that TOGT inhibition is significant among plants of the T1 progeny and show the efficiency of the antisense strategy, even after induction of the endogenous Togt genes.

TOGT Downregulation Leads to Decreased Scopoletin UGT Activity, Decreased Scopolin and Scopoletin Contents, and Alteration of the Blue Fluorescence Induced by Inoculation with Tobacco mosaic virus

Togt genes are induced rapidly in tobacco after the onset of the HR triggered by Tobacco mosaic virus (TMV) or a fungal elicitor (Fraissinet-Tachet et al., 1998), whereas no increase in TOGT is observed in TMV-infected leaves of susceptible tobacco cultivars (J. Chong, R. Baltz, and P. Saindrenan, unpublished results). Thus, TMV-infected plants of the resistant tobacco cv Samsun NN were used as the model system to study the role of TOGT during the induction of defense responses. We determined that TOGT inhibition in antisense adult plants inoculated with TMV was of the same magnitude as the inhibition measured in SA-treated plantlets (Figures 3C and 3D). Levels of residual TOGT protein after TMV inoculation were 7 to 14% and 28 to 30% of those of control plants for the AS 14 and AS 25 lines, respectively (Figures 3C and 3D).

Because scopoletin and SA were shown to be a good and a poor in vitro substrate of recombinant TOGT, respectively (Fraissinet-Tachet et al., 1998), UGT activities were measured in control and antisense plants with scopoletin and SA as substrates. In control plants, scopoletin UGT activity exhibited a basal level in mock-inoculated leaves (Figure 4A) and was fourfold higher after TMV inoculation (Figure 4A). We found decreased UGT activity relative to controls with scopoletin as substrate in AS 14 and AS 25 lines in both H2O- and TMV-treated plants (Figure 4A). In TMV-inoculated leaves, the average scopoletin UGT activity was 33 and 43% of that of controls for the AS 14 and AS 25 lines, respectively. However, the induction factor between mock- and TMV-inoculated plants remained the same in antisense plants compared with controls (Figure 4A).

UGT activity toward SA could be separated into two activities: one forming the SA β-O-glucoside (SOGT) and one forming the salicyloyl Glc ester (SEGT). SOGT activity was very low in healthy plants and was strongly induced by TMV inoculation (Figure 4B), whereas SEGT activity displayed a high basal level in noninfected plants and was virtually the same after infection (Figure 4C). Neither SOGT nor SEGT activity was affected substantially in antisense plants relative to empty-vector controls (Figures 4B and 4C). The activity of Phe ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway, also was induced to the same extent in the antisense and control lines (Figure 4D).
We further examined the effects of TOGT activity inhibition on the levels of soluble free and conjugated phenolic compounds in TMV-inoculated leaves 4 days after inoculation. Preliminary HPLC analyses of total soluble phenolic compounds coupled to fluorescence and UV light detection revealed that a peak corresponding to scopolin was reduced in the antisense lines. Scopolin and scopoletin thus were quantified more precisely in populations of plants from antisense and control lines (Figures 5A and 5B). In mock-inoculated control plants, levels of both scopolin and scopoletin were low (0.73 and 0.02 μg/g fresh weight, respectively). Mock-inoculated antisense transformants showed a twofold decrease in scopolin content compared with control plants (data not shown). In TMV-infected leaves of control plants, scopolin and scopoletin contents were maximal 4 days after inoculation, after the appearance of necrotic lesions.

At the same time, plants of both the AS 14 and AS 25 lines displayed a significant (threefold to fourfold) decrease in scopolin amounts (Figure 5A). Unexpectedly, the pool of the aglycon scopoletin also was reduced in the inhibited plants (Figure 5B). As with scopolin, scopoletin content was threefold to fourfold lower in the antisense lines relative to controls. By contrast, levels of SA glucosides (SA β-O-D-glucoside and salicyloyl Glc ester) after infection were not affected significantly in TOGT-inhibited plants (Figure 5C), and

![Figure 3. Analysis of TOGT Inhibition and of Typical Defense Markers in TOGT Antisense Plants Inoculated with TMV.](image)

Total proteins (5 μg) from TMV-inoculated leaves were analyzed by protein gel blotting. Each lane represents an individual T1 plant of the control lines (C1 and C4, transformed with the empty vector) and the AS 14 and AS 25 lines (transformed with Togt in the antisense orientation).

(A) Protein gel blot analysis with anti-TOGT and anti-COMT II sera 3 days after inoculation.

(B) Protein gel blot analysis with anti-TOGT and anti-β-1,3-glucanase sera 7 days after inoculation. Anti-β-1,3-glucanase serum recognizes three isoforms, PR-2, PR-N, and PR-O.

(C) Relative intensity of the TOGT and COMT II protein bands as shown in (A) after scanning the protein gel blot. The mean TOGT and COMT II levels in control lines were set to 100%. Values are means ± SE of TOGT and COMT II levels measured in three to four independent plants of the same line.

(D) Relative intensity of the TOGT and PR-2, PR-N, and PR-O protein bands as shown in (B) after scanning the protein gel blot. The mean TOGT and PR-2, PR-N, and PR-O levels in control lines were set to 100%. Values are means ± SE of TOGT and PR-2, PR-N, and PR-O levels measured in three to five independent plants of the same line.

![Figure 4. Glucosyltransferase and PAL Activities in TOGT-Inhibited Plants.](image)

Activities were measured in empty-vector (C1) and antisense (AS 14 and AS 25) lines 4 days after H2O (white) or TMV (black) inoculation. Values are means ± SE of activities measured in six plants from the same line. The experiment was repeated twice with similar results.

(A) Glucosyltransferase activity forming scopolin.

(B) Glucosyltransferase activity forming SA β-O-D-glucoside.

(C) Glucosyltransferase activity forming salicyloyl Glc ester.

(D) PAL activity.
A Tobacco UGT Involved in Defense Responses

no difference in free SA content between the different lines was observed (data not shown). Chlorogenic acid also accumulated to the same amounts in control and antisense plants (Figure 5D). The contents of ferulic acid and p-coumaric acid, which also are in vitro substrates of TOGT esterified to the cell wall, were analyzed after mild alkaline hydrolysis. However, no differences were found concerning these parietal components between control and TOGT-inhibited plants (data not shown).

Three to 4 days after TMV inoculation, lesions of control plants observed under UV light were surrounded by a ring of cells characterized by bright blue fluorescence (Figures 6A and 6B). Recently, it was shown that scopolin and scopoletin, which accumulate to high levels during the necrotic response of tobacco to TMV, probably are the major compounds responsible for the blue fluorescence observed under UV light (Costet et al., 2002). Compared with controls, the blue fluorescence around the lesions in antisense plants was reduced dramatically and appeared more diffuse (Figures 6A and 6B). This phenotype demonstrates that the reduction of scopolin and scopoletin contents is reflected by an alteration of the blue fluorescence characterizing living cells adjacent to TMV lesions.

Downregulation of TOGT by Antisense Expression Weaks Resistance to TMV

To gain further insight into the role of TOGT in defense response and pathogen resistance, we investigated the effect of antisense transformation on the local lesion response induced by the infection of Samsun NN tobacco with TMV.
After TMV inoculation, the leaves of control and antisense lines were scored for disease symptoms (i.e., necrotic lesion size and lesion number). With regard to lesion number, we found no difference between the empty vector–transformed and antisense populations (data not shown). The lesions also appeared at the same time (from 36 to 48 hr) in both populations and were indistinguishable in size between 40 and 72 hr after inoculation. By contrast, the lesion size was increased significantly in antisense plants compared with controls by 6 to 8 days after inoculation (Figure 7A).

Figures 7B and 7C show the distribution of lesion sizes 7 days after inoculation for the empty-vector controls and for the AS 14 and AS 25 lines. A mean diameter of 1.92 ± 0.28 mm was measured for the lesions of the empty vector–transformed population. For the AS 14 line, which displayed a 90% reduction in TOGT levels, the mean lesion diameter was 2.45 ± 0.33 mm, which corresponds to a statistically significant 28% increase in lesion diameter or a 63% increase in lesion surface relative to control plants (Figure 7B). An increase of 23% in lesion diameter was measured for the AS 25 line (Figure 7C).

Because lesion size is thought to reflect its viral content, we compared the amounts of TMV present in individual necrotic lesions of antisense and empty-vector plants. TMV amounts measured by ELISA in lesions of antisense plants were approximately twofold higher than those measured in lesions of control plants (Figure 7D). Together, these results clearly demonstrate that blocking Togt expression weakens the resistance to TMV infection and strongly suggest an active role for TOGT in the mechanism of resistance to TMV.

To determine if other components of defense responses might be modulated by TOGT inhibition, we tested by protein gel blot analysis the expression of two defense markers induced during the resistance response to TMV, the class II O-methyltransferase (COMT II) (Pellegrini et al., 1993) and acidic β-1,3-glucanases (PR-2, PR-N, and PR-O) (Kauffmann et al., 1987) (Figure 3). Figures 3A and 3C show the inhibition of TOGT accumulation in antisense lines 3 days after inoculation with TMV. COMT II accumulation was not affected significantly in either the AS 14 or the AS 25 line (Figures 3A and 3C). Acidic β-1,3-glucanases also accumulated to similar levels in leaves of control and antisense plants 7 days after TMV inoculation (Figures 3B and 3D). Thus, the effect of TOGT downregulation is specific and does not affect the intensity of expression of two typical markers associated with the HR, reflecting early (COMT II) and late (PR-2, PR-N, and PR-O) events of defense responses, respectively.

Scopoletin Inhibits TMV Replication in Infected Tobacco Protoplasts

To determine if decreased virus resistance could be related to scopolin/scopoletin depletion, we investigated whether this hydroxycoumarin could inhibit TMV replication in tobacco protoplasts. Tobacco BY2 protoplasts were treated with scopoletin or H₂O immediately before their inoculation with TMV. The scopoletin concentrations used did not affect protoplast viability. The accumulation of TMV genomic RNA was examined 48 hr after inoculation by RNA gel blot analysis using a cDNA probe specific for full-length TMV RNA (Figure 8). Treatment of protoplasts with scopoletin caused a significant reduction in the accumulation of TMV genomic RNA compared with treatment with H₂O (Figure 8A). Quantification of the signal corresponding to TMV full-length RNA (Figure 8B) showed that the inhibitory effect of scopoletin was dose dependent: relative amounts of TMV genomic RNA were 57 and 25% of those of controls with 0.1 and 0.5 mM scopoletin, respectively (Figure 8B).

TMV-Induced ROI Accumulation Is Enhanced in TOGT-Inhibited Plants

We showed in a previous study that scopoletin is synthesized in tobacco cells upon elicitation of defense responses and transported as a glucoside to the extracellular space, where it is released in its free form by β-glucosidases (Chong et al., 1999). This extracellular free scopoletin could act as a direct scavenger of H₂O₂ deriving from the oxidative burst (Chong et al., 1999). To determine if decreased scopoletin and scopoletin contents could enhance ROI accumulation in planta, we assayed tobacco plants for the production...
of ROIs in response to TMV infection. We used the sensitive fluorophore dichlorofluorescin (DCFH), which is oxidized to the highly fluorescent dichlorofluorescein (DCF) in the presence of H₂O₂ and peroxidases, to measure increases in ROIs (Schopfer et al., 2001).

ROIs were measured on foliar discs centered on a single TMV lesion and including 2 mm of surrounding living tissue. To estimate specifically the increase in extracellular ROIs, we used the procedure developed by Schopfer et al. (2001) (see Methods). The kinetics of DCFH oxidation were first assayed with TMV-inoculated discs of wild-type Samsun NN plants 60 hr after inoculation. To confirm that DCF fluorescence was indicative of the oxidative burst, we used several ROI inhibitors. Table 1 shows that DCFH oxidation by TMV-inoculated leaf discs could be inhibited by a general antioxidant (Na-ascorbate), by a H₂O₂ scavenger (catalase), and by peroxidases and NADPH oxidase inhibitors (NaN₃ and diphenylene iodonium, respectively). The kinetics of DCFH oxidation then were assayed with TMV-inoculated leaf discs of antisense and control plants 60 and 96 hr after inoculation as well as with discs of water-inoculated leaves (Figure 9). No ROI release was measured with discs from non-treated plants (data not shown).

In mock-inoculated discs, an increase in ROIs was measured, as a consequence of the effect of wounding with the abrasive, but no difference in ROI accumulation was detected between antisense and control plants (Figure 9). TMV inoculation of control plants induced a greater extracellular ROI accumulation than H₂O₂ treatment (Figure 9). However, compared with control plants, the extracellular ROI increase reflected by DCFH oxidation was significantly higher when measured on TMV-inoculated leaf discs from antisense plants (Figure 9). The slopes of curves of DCF fluorescence showed 1.5- and 2.2-fold increases in DCFH oxidation compared with controls in the incubation medium of TOGT-depleted lesions at 60 and 96 hr after TMV inoculation.

Figure 7. Resistance to TMV is Weakened in TOGT Antisense Plants. (A) Aspect of lesions under visible light 7 days after TMV inoculation. CT, empty vector-transformed plant. Bar = 2 cm.

(B) and (C) Distribution of lesion sizes for the AS 14 and control populations (B) and for the AS 25 and control populations (C). Two leaves of plants from control lines (C1 and C4; black bars) and from antisense lines (AS 14 and AS 25; hatched bars) were inoculated with TMV and scored for lesion diameter on day 7. For each antisense and control line, 10 plants were inoculated and ~1000 lesions were scored. Values are mean lesion sizes ±SE for the number of lesions measured. The experiment was repeated three times with similar results. The distributions of lesion sizes were normal, allowing the comparison of the mean lesion diameter for each population. The mean lesion sizes of the AS 14 and AS 25 populations were significantly different from that of the empty-vector population (Student’s t test; P < 0.01).

(D) Effect of Togt antisense transformation on the amount of TMV measured in individual 7-day-old necrotic lesions. TMV was quantified by the double antibody sandwich form of ELISA (see Methods). Values are mean TMV amounts ±SE measured in 30 lesions of control and TOGT-inhibited AS 14 and AS 25 populations.
respectively (Figure 9). Hence, the most pronounced differences in ROI accumulation between antisense and control plants were measured 96 hr after virus inoculation. Remarkably, they were correlated with the differences in blue fluorescence that developed around lesions and with the changes in scopoletin pools that were observed concomitantly.

**DISCUSSION**

**TOGT Is Involved in Scopoletin Glucosylation**

By regulating the solubility, biological activity, and transport of compounds within the cell, glucosyltransferases are crucial in the maintenance of cellular homeostasis. Despite the number of plant secondary product UGTs, the direct demonstration of their function by altering their expression in transgenic plants has been reported only rarely. Here, we show that inhibition of TOGT, a phenylpropanoid glucosyltransferase acting mostly on hydroxycoumarins in vitro (Fraissinet-Tachet et al., 1998), suppressed the accumulation of scopolin by 70 to 75% in tobacco reacting hypersensitively to TMV (Figure 5). Moreover, the close correlation between scopolin depletion and reduced scopoletin UGT activity in the inhibited lines strongly supports the requirement of TOGT for scopoletin glucosylation. Interestingly, the levels of the free form (scopoletin) were reduced to the same extent as the levels of scopolin in inhibited transformants, although one would expect that the inhibition of the conjugation step would trigger the accumulation of the corresponding free form.

Several hypotheses could explain this unexpected reduced scopoletin content. First, it is known that phenolic compounds cannot accumulate to high levels as aglycons in plant cells, because they are very reactive and subject to oxidation. Scopoletin is a reactant of peroxidases in vitro and in vivo (Chong et al., 1999), and it is likely that compromising scopoletin glucosylation could enhance its degradation by cellular oxidases. Second, one part of the scopoletin pool may arise from the cleavage of the glucosylated form (scopolin) by β-glucosidases (Figure 1), as proposed recently for elicited tobacco cells (Chong et al., 1999). The mobilization of the free forms from a pool of conjugated phenolic compounds also was reported in the case of resveratrol glucoside in transgenic alfalfa (Hipskind and Paiva, 2000) and for SA O-D-glucoside in tobacco (Seo et al., 1995). Third, TOGT inhibition could alter metabolic flux toward the conjugated form (scopolin), resulting in the feedback inhibition of upstream enzymes of scopoletin biosynthesis.

Studies of transgenic plants downregulated for COMT I, an enzyme involved in lignin biosynthesis, revealed that

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**Table 1.** Effect of ROI Inhibitors on ROI Production by TMV-Inoculated Leaf Discs

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<tr>
<th>Inhibitor</th>
<th>ROI Production (%)</th>
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<tr>
<td>None</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Catalase (100 μg/ml)</td>
<td>46 ± 8</td>
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<tr>
<td>Na-ascorbate (10 mM)</td>
<td>41 ± 3</td>
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<tr>
<td>DPI (100 μM)</td>
<td>55 ± 1</td>
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<tr>
<td>NaN3 (1 mM)</td>
<td>50 ± 1</td>
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The ROI-dependent oxidation of DCFH by 20 TMV-inoculated leaf discs from Samsun NN tobacco 60 hr after inoculation was determined after an incubation period of 30 min with DCFH in the presence of various inhibitors. The lesions were preincubated in the inhibitor solution for 15 min before the addition of DCFH. DPI, diphenylene iodonium.
The experiments described here also present direct evidence of the important role of a UGT in virus resistance. We show that a deficiency in TOGT decreased the resistance of tobacco to TMV infection, as determined by the significant increase in lesion surface (+60%) and by the twofold increase in the amount of TMV accumulation in the necrotic lesions. The phenotype of the antisense lines could be associated specifically with TOGT depletion. Indeed, the antisense plants did not seem to be affected significantly in mounting other typical defense reactions, such as the induction of enzymes involved in lignification (COMT II) and glucosylation of hydroxycinnamic acid derivatives is thought to activate the acyl moiety for trans-esterification into the cell wall followed by cross-linking into phenolic polymers (Hahlbrock and Scheel, 1989). However, TOGT inhibition did not affect the wall-bound phenolic profiles of TMV-inoculated leaves after mild alkaline hydrolysis, suggesting that TOGT would not be involved in the production of cell wall impregnation precursors.

In conclusion, our data clearly demonstrate the function in planta of TOGT in scopoletin glucosylation and further indicate that although SA was found to be a strong inducer of Togt expression (Horvath and Chua, 1996; Fraissinet-Tachet et al., 1998), this UGT apparently is not required for the regulation of the SA signal during plant defense responses. Nevertheless, it should be noted that even when TOGT expression was nearly suppressed in the AS 14 line, the scopoletin content was not depleted completely. Similarly, a basal level of scopoletin UGT activity remained in antisense plants. These results indicate that other UGTs may exist in tobacco that are active toward scopoletin, although perhaps with less affinity than TOGT. In support of this idea, a novel glucosyltransferase from tobacco with 45% similarity to TOGT that preferentially glucosylates flavonoids in vitro and to a lesser extent coumarins such as scopoletin was characterized recently (Taguchi et al., 2001).
the accumulation of pathogenesis-related proteins (β-1,3-glucanases). However, if we consider that the number of TMV-stimulated cells by day 7 after inoculation is enhanced to some extent by the larger size of the lesions in the antisense lines, the accumulation of β-1,3-glucanases should be higher in these plants, but this augmentation probably is too slight to be revealed by protein gel blot analysis.

In tobacco reacting hypersensitively to TMV, the cells beyond necrotic lesions still contain infectious virus particles (Konate et al., 1982). This observation shows that hypersensitive cell death is not sufficient to restrict pathogen spread and that defense responses induced in living cells that surround HR cells are required for full pathogen containment. Dorey et al. (1997) showed that during the HR induced by a fungal elicitor, a low and transient increase was found in total scopoletin in HR cells, whereas a strong accumulation was measured in cells adjacent to the necrosis. Our work strongly suggests that decreased resistance to TMV in antisense lines is a consequence of reduced scopolin and scopoletin contents, principally in living cells surrounding the necrotic zone, which contribute to pathogen spread limitation.

There is substantial evidence that scopolin and scopoletin play an important role in plant disease resistance. Earlier studies demonstrated that scopolin accumulates only in TMV-resistant cultivars after inoculation (Tanguy and Martin, 1972). Moreover, it has been shown that scopoletin possesses antimicrobial activity in vitro and therefore can be considered a phytoalexin (Ahl-Goy et al., 1993; Kuc, 1995). Increased constitutive levels of scopolin and scopoletin also were measured in a Nicotiana hybrid that is resistant to viral, bacterial, and fungal diseases (Ahl-Goy et al., 1993). Phytoalexins generally are believed to exert their antimicrobial activity toward fungi and bacteria. However, our work provides evidence that exogenous scopoletin, at concentrations similar to those of total scopoletin around HR lesions (~250 μM), was able to inhibit TMV replication in tobacco protoplasts. Thus, scopoletin accumulation could be part of the mechanism of virus restriction in plants.

It is known that phenolic compounds display antiviral activity, especially against animal viruses. For example, phenolic moieties, and particularly compounds containing 4-hydroxycoumarin residues, are inhibitors of the Human immunodeficiency virus integrase required for virus replication (Mazumder et al., 1996). A few studies have demonstrated the antiviral activity of phenylpropanoids toward plant viruses. However, a range of flavonoids inhibit the infectivity of TMV (French et al., 1991). SA also was shown to interfere with TMV replication in compatible interactions with tobacco (Chivasa et al., 1997) and to inhibit the replication of Alfalfa mosaic virus in cowpea protoplasts (Hooft van Huijstduijnen et al., 1986). Thus, the higher virus content in TOGT-inhibited plants may be a consequence of the reduction of the antiviral scopoletin pool, which would allow enhanced TMV replication and accumulation in cells adjacent to HR lesions.

**Scopoletin May Represent an Antioxidant in Living Cells Surrounding HR Lesions**

Besides the possible antiviral role of scopoletin investigated in this study, this molecule likely represents a potent antioxidant in plant defense responses (Chong et al., 1999), together with scopolin, which can act as a source of scopoletin after its hydrolysis by β-glucosidases. ROIs (O2•− or H2O2) generated via the oxidative burst have been proposed to play a central role in the development of host cell death during the HR (Dangl et al., 1996). Indeed, programmed cell death during the HR was enhanced in transgenic plants with reduced levels of ascorbate peroxidase or catalase, which are unable to properly remove ROIs (Mittler et al., 1999).

It also has been proposed that high levels of ROIs induce cell death typical of HR, whereas lower levels, such as those found at the lesion margin, activate the transcription of genes that code for enzymes involved in the resistance to oxidative damage (e.g., glutathione S-transferases and glutathione peroxidases) (Jabs et al., 1996; Lamb and Dixon, 1997). This arrangement would regulate ROI accumulation, limit the extent of necrosis, and maintain the capacity of surrounding cells to deploy transcription-dependent defenses (Lamb and Dixon, 1997). Thus, it was reported in tobacco that catalase expression and activity decreased in HR cells, whereas catalase activity was induced in living cells beyond HR lesion (Dorey et al., 1998).

Many hydroxycinnamic acid derivatives also can act as scavengers of O2•− and ascorbate-dependent peroxidases acting on phenolic substrates may remove H2O2 from the apoplasm (Lamb and Dixon, 1997). Our study suggests the hypothesis that scopoletin, together with other antioxidant systems, plays a role in the regulation of ROI accumulation in living cells surrounding necrotic lesions, either as a substrate of peroxidases or as a direct ROI scavenger. This idea is supported by ROI measurements on TMV infected tissues, which revealed a more intense ROI accumulation relative to controls, in antisense plants also characterized by a reduction of antioxidant scopol in and scopoletin pools.

Interestingly, the role of phenolic compounds as endogenous antioxidants has been emphasized in recent work demonstrating that the inhibition of phenolic acid metabolism triggered premature cell death in tobacco leaves, suggesting that the oxidation of phenolic compounds is part of a protective mechanism that reduces the impact of increased ROI levels during senescence (Tamagnone et al., 1998). Moreover, transgenic tobacco plants that accumulate tetrapyrole intermediates were found to accumulate scopoletin constitutively in their leaves (Mock et al., 1999). Because it is known that the toxicity of tetrapyrroles is exerted via ROIs (Mock et al., 1999), scopoletin accumulation could represent a means to withstand ROIs in these plants. Our results obtained with TOGT transgenic plants further support the idea of the participation of antioxidant hydroxycoumarins in the control of ROI accumulation during plant-pathogen interactions.
H$_2$O$_2$ has been proposed to act as a signal that activates plant defense responses (Lamb and Dixon, 1997) and resistance in the case of the interaction with avirulent bacteria (Alvarez et al., 1998). The enhanced ROI accumulation in TOGT-downregulated lines may have been expected to enhance the expression of host defense responses and virus resistance. However, it should be noted that H$_2$O$_2$ contents may not be limiting for the induction of defense responses in wild-type plants. Thus, the 1.5- to 2-fold increase in H$_2$O$_2$ observed in antisense plants does not necessarily trigger an increase in defense gene expression. Moreover, because TOGT is likely to play a role in living cells adjacent to HR lesions, the increase in ROI accumulation in inhibited plants does not contradict the decreased TMV resistance. Indeed, ROI accumulation in neighboring cells must be controlled tightly to limit the effects of the oxidative stress and to allow the full expression of defense responses toward TMV.

**Conclusion**

Our work provides clear evidence of the biological role of a phenylpropanoid UGT in plant defense responses and demonstrates the importance of TOGT in the control of free and conjugated scopoletin pools. This study also sheds new light on the importance of glucosylation in the accumulation of phenylpropanoids that play a dual role in plant defense, as both antiviral agents and antioxidant compounds involved in the maintenance of the cellular redox state. Two nonexclusive hypotheses could explain the phenotype of TOGT antisense lines after virus infection. The greater number of dead cells and the higher virus content reflected by the enlarged lesions may be a consequence of increased virus replication and/or enhanced oxidative stress.

**METHODS**

**Chemicals**

Salicylic acid (SA), scopoletin, chlorogenic acid, and UDP-Glc were from Sigma Aldrich Chimie (St. Quentin-Fallavier, France). SA β-D-glucoside and salicyloyl Glc ester were kindly provided by Prof. S. Tanaka (University of Kyoto, Japan). Scopolin was obtained enzymatically with recombinant tobacco SA- and pathogen-inducible UGT (TOGT) (Fraissinet-Tachet et al., 1998). 7-14C-Salicylic acid (2 GBq/mmol) and UDP-β-U-14C-Glc (10.6 GBq/mmol) were obtained from DuPont–New England Nuclear (Boston, MA). Dichlorofluorescin-diacetate (DCFH-DA; Sigma) was dissolved in 100% ethanol to produce a 100 mM stock.

**Vector Construction**

All DNA recombinant procedures were performed essentially as described by Sambrook et al. (1989). The binary vector pFB8 (Atanassova et al., 1995) contains a bacterial neomycin phosphotransferase II gene as a plant-selectable kanamycin resistance marker. The Togt1 full-length cDNA (1428 bp) (Fraissinet-Tachet et al., 1998) was isolated from a pGEM-T vector (Promega, Madison, WI). A Togt polymericase chain reaction fragment was generated using primers 5'-GTCCCCGGATCTAGACATGGGTACGTCACCATTTTTC3' and 5'-GCCGCTCGAGCTCTTAATGACCAGTAGAAGATG-3' (priming at positions +1 and +1428 relative to the ATG start codon of Togt1, respectively), introducing a Xhol site at the 3' end and a BamHI site at the 5' end of the coding region. The fragment was cloned in the antisense orientation downstream from the 420-bp 3SS RNA promoter of Cauliflower mosaic virus in the Xhol-BamHI sites of pFB8 vector. The binary vector was mobilized into Agrobacterium tumefaciens strain LBA4404 by electroporation. The integrity of the vector was verified directly by polymerase chain reaction on Agrobacterium.

**Plant Transformation and Regeneration**

Tobacco (Nicotiana tabacum cv Samsun NN) was transformed via Agrobacterium and regenerated by a modification of the leaf disc method as described previously (Atanassova et al., 1995). Forty independent transformants were regenerated as well as 20 independent plants transformed with the empty (without insert) vector as controls. The primary transformants (T0) were first screened by protein gel blot analysis for reduced TOGT protein levels in plantlet leaves treated with SA (data not shown). Plantlets of the inhibited lines were transferred to the greenhouse and cultivated in soil under a light/dark period of 16/8 hr at 22 ± 4°C until flowering. Plants were allowed to self-pollinate, and T1 seeds were harvested and selected further on germination medium containing kanamycin. The T1 progeny were used in all of the experiments described.

**Infection of Tobacco Leaves and Protoplasts with Tobacco mosaic virus**

For plant infection, 10-week-old transgenic plants cultivated in the greenhouse were inoculated by rubbing fully expanded leaves with a suspension of Tobacco mosaic virus (TMV) (U1 strain; 0.1 μg/mL) in water containing cello powder as an abrasive. Leaves rubbed with an aqueous cello suspension were used as wounded controls. Leaves were harvested at different times after inoculation, frozen in liquid N$_2$, and stored at −80°C.

For protoplast infection, the tobacco BY2 cell line was cultured as described by Nagata et al. (1992). Protoplasts were prepared as described by Gaire et al. (1999) and resuspended in 3.6 mM 2-(N-morpholino)-ethanesulfonic acid, pH 5.5, containing 0.45 M mannitol and 0.1 mM CaCl$_2$ (solution A). Scopolin was dissolved in DMSO to produce a stock solution (1 M) and was added to protoplasts to a final concentration of 0.1 or 0.5 mM immediately before electroporation. Control protoplasts were treated with DMSO alone at the same concentration.

Protoplasts (1 × 10$^6$ in 0.5 mL) were electroporated with a Gene Pulsor (Bio-Rad, Hercules, CA) in a 0.4-cm-path-length cuvette at 0.18 kV, 100 μF, and 125 μF using 1 μg of purified TMV (U1 strain). After electroporation, 1 mL of solution A was added, and the protoplasts were incubated for 30 min on ice before sedimentation. Protoplasts then were cultured at 25°C in 1.5 mL of BY2 culture medium plus 0.45 M mannitol in the presence or absence of scopolin in 35-mm-diameter Petri dishes containing a bottom layer of 1%
agarose. Protoplasts were collected carefully after 48 hr by centrifugation at 40g.

SA Treatment

Leaves of 6-week-old in vitro–grown plants were excised, allowed to imbibe a solution of 1 mM potassium salicylate, pH 6.5, and kept under light at 25°C for 16 hr. SA-treated leaves were frozen in liquid N2 and stored at −80°C.

Protein Gel Blot Analysis

Foliar explants were harvested from 6-week-old in vitro–regenerated T1 plants treated with SA or from 10-week-old TMV-inoculated T1 plants cultivated in the greenhouse. Samples (0.5 g) were ground in liquid N2 and extracted with 1.5 mL of 200 mM potassium phosphate buffer, pH 6.0, containing 28 mM β-mercaptoethanol and 1% polyvinylpyrrolidone. SDS-PAGE was performed according to standard procedures. Proteins were blotted onto Immobilon P membranes (Millipore, Bedford, MA) as described previously (Baillieul et al., 1995). Detection was realized with the Immun-Star Chemiluminescent Kit (Bio-Rad).

Polyclonal antibodies raised against recombinant TOGT produced in Escherichia coli (Fraissinet-Tachet et al., 1998) were used at a dilution of 1:10,000. Relative TOGT protein amounts were quantified by scanning the protein gel blot and measuring the relative intensity of the 52-kD band corresponding to TOGT using MacBAS software (Fuji, Tokyo, Japan). Polyclonal antibodies raised against class II O-methyltransferase (Pellegrini et al., 1993) and against three acidic β-1,3-glucanase isoforms (PR-2, PR-N, and PR-O) (Kauffmann et al., 1987) purified from tobacco were used at dilutions of 1:5000 and 1:2000, respectively.

Glucosyltransferase and Phe Ammonia-Lyase Activity Measurements

Phe ammonia-lyase activity was extracted and measured as described by Pellegrini et al. (1993). For UGT activity, samples (1 g) were extracted as described by Chong et al. (1999). Scopolin UGT and SA UGT activities were performed with 30 μL of concentrated extract in 50 μL of reaction mixture containing 100 μM scopolin, 110 μM UDP-14C-Glc (10.6 GBq/mmol) or 180 μM 7-14C-SA (2 GBq/ mmol), and 200 μM unlabeled UDP-Glc. Incubations were terminated by the addition of 50 μL of methanol (MeOH). Reaction products were analyzed on a 0.25-mm silica gel thin layer chromatography (TLC) plates according to Fraissinet-Tachet et al. (1998). Radioactivity on TLC plates was visualized and quantified with a Bio-Imager analyzer (Fuji). Reaction products were identified by a combination of cochromatography on TLC plates with authentic compounds and analysis by HPLC with a photodiode array detector (Millenium software; Waters, Milford, MA) as described previously (Fraissinet-Tachet et al., 1998).

Determination of Phenolic Compounds

Plant material (0.5 g) was extracted twice with 1 mL of 90% (v/v) MeOH. Aqueous MeOH was removed under N2, and the dried residue was dissolved in 300 μL of 5% acetonitrile in 25 mM NaH2PO4, pH 3.0, before HPLC analysis. 4-Methylumbelliferone (1 nmol) was added to each sample before extraction as an internal standard. HPLC analysis was performed on a C18 Nova Pak column (particle size, 5 μm; 4.6 × 150 mm; Waters) using a gradient of 25 mM CH3CN in NaH2PO4, pH 3, at a flow rate of 1 mL/min. The gradient was 5 to 22% for 35 min and then 22 to 80% for 1 min. Scopolin and scopoletin (retention times, 16.5 and 26.5 min, respectively) were detected by fluorescence (excitation, 290 nm; emission, 402 nm). Chlorgenic acid (retention time, 15 min) was detected by UV light spectrophotometry at 280 nm. Free and conjugated SA were extracted and detected by fluorescence (excitation, 315 nm; emission, 405 nm) as described by Baillieul et al. (1995). Identification of the compounds was further based on cochromatography with authentic standards coupled to a photodiode array detector (detection between 230 and 400 nm; Waters Millenium software). Compounds were quantified by comparison with reference compounds.

Extraction of Total RNA from Infected Tobacco Protoplasts and Detection of Viral RNA

A cDNA probe specific for the TMV genome was used to detect TMV genomic RNA synthesized in protoplasts. The M11-Nhel cDNA fragment (1111 bp) of the TMV 30 B vector (Shivprasad et al., 1999) corresponding to a fragment of the gene encoding the 126-kD protein of the replicase complex was labeled by random priming with [32P]-dCTP using Ready-To-Go labeling beads from Amersham Pharmacia Biotech according to the supplier’s instructions. Protoplasts (1 × 106) were disrupted in 500 μL of 100 mM Tris-HCl, pH 8, 100 mM LiCl, 10 mM EDTA, and 0.1% SDS. Total RNA was extracted twice with phenol-chloroform (1:1, v/v) and precipitated for 12 hr on ice with 0.2 M LiCl. The pellet was washed twice with 70% ethanol, air-dried, and resuspended in H2O. Total RNA (10 μg) was separated by electrophoresis on a 1% formaldehyde-agarose gel and blotted for 12 hr with 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) to a Hybond N+ membrane. The membrane was hybridized successively according to standard protocols (Sambrook et al., 1989) with the 126-kD probe and with an Arabidopsis 25S rRNA probe provided by the ABRC (Columbus, OH).

Quantification of TMV in Plant Tissue by ELISA

Discs of tissue were cut to a diameter of 6 mm from the center of individual lesions of 7-day-inoculated tobacco leaves. Individual lesions were extracted with 250 μL of 100 mM potassium buffer, pH 6, containing 1% polyvinylpyrrolidone. After centrifugation, the supernatant was used for TMV detection. Approximately 30 lesions were tested for each line of antisense and control plants.

The “double antibody sandwich” form of ELISA was used. Polystyrene microtiter plates (microlon 96K; Greiner, Frickenhausen, Germany) were coated overnight with a solution of chicken anti-TMV IgG (2 μg/mL) in 0.5 M sodium carbonate, pH 9.8. Saturation of aspecific sites was achieved with 1% BSA in PBS-Tween for 1 hr at 37°C. Different dilutions in PBS-Tween of the crude extract of lesions (1:20, 1:100, and 1:500) were kept in the wells for 2 hr at 37°C. Plates were incubated further with the second antibody (i.e., rabbit anti-TMV IgG; 10 μg/mL) for 2 hr. Goat anti-rabbit antibodies coupled to alkaline phosphatase (2 μg/mL; Dako, Glostrup, Denmark) then were added for 1 hr. Alkaline phosphatase activity was revealed by adding p-nitrophenylphosphate (1 mg/mL) in 0.1 M diethanolamine buffer, pH 9.8. After incubation for 20 min at room temperature, the absorbance
was measured at 405 nm. Plates were washed three times between each step with PBS buffer containing 0.05% Tween. TMV concentration in each extract was determined by comparison with a standard curve obtained with solutions of 1 to 250 ng/mL purified TMV.

In Vivo Detection of H2O2 in Plants

Leaves of 10-week-old antisense and control plants were inoculated with TMV on one half and with water on the other half. Tissue discs were cut to a diameter of 6 mm from the center of individual lesions 60 or 96 hr after inoculation. Discs of the same size from water-inoculated half leaves were used as wounded controls. Plants were maintained in the dark for 2 hr before harvest to obtain low basal levels of reactive oxygen intermediates (ROIs). DCFH can be introduced into the cell as esterified DCFH-DA, where it is deacetylated by endogenous esterases and trapped as DCFH, a nonfluorescent compound. To measure increases in extracellular ROIs, the method developed by Schopper et al. (2001) was used. This method consists of deacetylating DCFH-DA to the membrane-impermeant DCFH before adding it to necrotic lesions incubated in phosphate buffer. In this way, the ROI-dependent oxidation of DCFH to dichlorofluorescein (DCF) reflects the extracellular oxidative burst and can be measured by the increase in DCF fluorescence in the liquid incubation medium of leaf discs.

A 10 mM solution of DCFH-DA in 20 mM potassium phosphate buffer, pH 6.0, was incubated in the dark for 40 min at 25°C with 2 g/L esterase (from hog liver; Sigma Aldrich Chimie) for deacetylation. Before ROI assays, foliar discs were preincubated for 1 hr in Petri dishes containing 20 mM potassium phosphate buffer, pH 6, to remove preformed ROIs. ROI assays then were performed by incubating 20 discs in 6 mL of 100 mM deacetylated DCFH in potassium phosphate buffer at 25°C on a shaker in darkness. At different times after the addition of DCFH, a 1-mL aliquot of the solution was removed, and the increase in fluorescence (excitation, 488 nm; emission, 525 nm) indicating the ROI-dependent oxidation of DCFH to DCF was measured at 405 nm. Plates were washed three times between each step with PBS buffer containing 0.05% Tween. TMV concentration upon elicitation of defense responses and role as salicylic acid precursors. Plant Physiol. 117, 1095–1101.


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Weakens Virus Resistance

Glucosyltransferase Reduces Scopoletin Glucoside Accumulation, Enhances Oxidative Stress, and

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