FEBS Letters 458 (1999) 204–208 FEBS 22597

An early salicylic acid-, pathogen- and elicitor-inducible tobacco glucosyltransferase: role in compartmentalization of phenolics and H_2O_2 metabolism

Julie Chong, Rachel Baltz, Bernard Fritig, Patrick Saindrenan*

Institut de Biologie Moléculaire des Plantes du CNRS, Université Louis Pasteur, 67000 Strasbourg, France

Received 31 May 1999; received in revised form 27 July 1999

Abstract Treatment of tobacco cell suspension cultures with a fungal elicitor of defense responses resulted in an early accumulation of the phenylpropanoid glucosyltransferase TOGT, along with the rapid synthesis and secretion of scopolin, the glucoside of scopoletin. Elicitor-triggered extracellular accumulation of the aglycone scopoletin and of free caffeic and ferulic acids could only be revealed in the presence of diphenylene iodonium, an inhibitor of extracellular H_2O_2 production. Our results strongly support a role for TOGT in the elicitor-stimulated production of transportable phenylpropanoid glucosides, followed by the release of free antioxidant phenolics into the extracellular medium and subsequent H_2O_2 scavenging.

Key words: Defense response; Phenylpropanoid; Hydroxycoumarin; Glucosylation; H_2O_2; *Nicotiana tabacum*

1. Introduction

Plant resistance to pathogen attack is often associated with the so-called hypersensitive response (HR), characterized by the rapid death of the first infected host cells and the restriction of pathogen spread. Among the early events underlying HR induction are the production of reactive oxygen intermediates (ROIs) such as hydrogen peroxide (H_2O_2), the insolubilization of cell wall proteins by peroxidative cross-linking and an increased metabolic flux through the phenylpropanoid pathway leading to deposition of wall-bound phenolics [1]. The phenylpropanoid pathway is part of general phenolic metabolism and provides the signal molecule salicylic acid (SA), the precursors for lignin biosynthesis and abundant conjugated hydroxycinnamic acids, such as chlorogenic acid which is ubiquitous in plants [2].

Two early SA-inducible tobacco genes (*Togt*) that are also induced by a fungal elicitor or during the HR of tobacco to tobacco mosaic virus (TMV) were recently shown to encode glucosyltransferases acting very efficiently on phenylpropanoid derivatives such as hydroxycinnamic acids and hydroxycoumarins [3]. The precocious expression of these primary-response genes suggests a role of their products in stress responses and in regulation of late gene expression [4].

*Corresponding author. Fax: (33) 3 88 61 44 42.
E-mail: sdrenan@medoc.u-strasbg.fr

Abbreviations: TOGT, tobacco glucosyltransferase; HR, hypersensitive response; ROIs, reactive oxygen intermediates; TMV, tobacco mosaic virus; UDPG:scopoletin GTase, UDP-glucose:scopoletin glucosyltransferase; DPI, diphenylene iodonium

Conjugation reactions catalyzed by glucosyltransferases are providing a means for regulating activities and subcellular localization of phytohormones [5]. Glucosylation of endogenous phenolic compounds results in their enhanced water solubility and is thought to protect the highly reactive phenolic hydroxyls against cellular oxidases. Furthermore, the formation of phenolic glucosides is important in their transport to the cell wall before incorporation of the phenolic moiety [2].

Scopoletin (6-methoxy-7-hydroxycoumarin) was shown to be one of the best in vitro substrates of TOGT proteins [3]. This fluorescent compound present in several plant species is known to accumulate in solanaceous plants upon infection and is generally considered to be an antimicrobial compound [6]. In many plants or cell suspensions, scopoletin occurs as its glucoside scopolin, which results from the glucosylation of the free form by endogenous glucosyltransferases [7]. Interestingly, scopoletin is a known reactant of peroxidases in vitro [8] and as such fulfills all the properties of ROI scavengers. *Togt* gene products are also active on hydroxycinnamic acids such as caffeic and ferulic acids, which are widely distributed in plants and known to display potent antioxidant properties [9].

Here, we report that in tobacco cell suspension cultures treated with a fungal protein elicitor, a simplified model system mimicking the HR in plants, TOGT may be involved in the formation and fate of scopolin, the glucoside of scopoletin. Elicitation of defense responses triggers an early production of H_2O_2 along with the rapid secretion of scopolin into the extracellular compartment. Evidence is provided that extracellular scopolin may be further hydrolyzed by a cell wall-associated β-glucosidase leading to the release of the aglycone scopoletin. During elicitation, this extracellular scopoletin as well as the other powerful antioxidant caffeic and ferulic acids are rapidly oxidized via an enzymatic reaction involving H_2O_2 consumption. Together, our results suggest that TOGT protein participates in the sequence of events leading to secretion of phenylpropanoid derivatives which are involved in the adjustment of the cellular redox state occurring in plants responding to stress.

2. Materials and methods

2.1. Chemicals

Caffeic acid, ferulic acid, scopoletin and diphenylene iodonium (DPI) were from Sigma (Germany). Scopolin was obtained enzymatically by incubating 10 μg recombinant TOGT in 100 mM potassium phosphate buffer pH 6 with 800 μM scopoletin and 400 μM UDP-glucose. Scopolin was identified as described previously [3].

2.2. Biological material and treatments

BY-cultured tobacco cells (derived from *Nicotiana tabacum* cv.

PII: S0014-5793(99)01154-0

Bright Yellow) were grown in MS medium [3]. Six-day-old BY cells were induced with 50 nM β-megaspermin, a proteinaceous HR-inducing elicitor isolated from *Phytophthora megasperma* [10]. BY cells and culture medium were collected separately by vacuum filtration. For inhibition experiments, 5 μM DPI was added 5 min before treatment with β-megaspermin.

2.3. Determination of scopoletin, scopolin and hydroxycinnamic acids

Cells (0.5 g) were extracted twice with 1 ml 90% MeOH. Aqueous MeOH was removed under nitrogen and the dried residue was dissolved in 200 μl MeOH before HPLC analysis. For identification of extracellular phenolic compounds, 2 ml of culture medium was passed through a C18 Sep-Pak cartridge (Waters, France) and phenolics were eluted with 0.1% HCl in MeOH. For routine quantification, the medium was acidified to 2 N HCl and split into two equal parts; one part was processed for analysis of free phenolics and extracted twice with 2 volumes of ether. The other part was hydrolyzed for 30 min at 80°C before extraction with ether and used for total scopoletin quantification. Ether phases were dried under nitrogen. Scopolin content was assigned as the difference between total and free scopoletin. 4-Methylumbelliferone (1 nmol) was added to each sample before extraction as internal standard. HPLC analysis was performed on a C18 Nova Pak column (Waters), using a gradient of CH_3CN (A) in NaH_2PO_4 25 mM pH 3 (B) at a flow rate of 1 ml/min. The gradient was 5% to 22% (A) for 35 min and then 22% to 80% (A) for 1 min. Scopoletin and scopolin were detected by fluorescence (λ_{ex} 290 nm, λ_{em} 402 nm), caffeic and ferulic acids by UV spectrophotometry at 320 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 compounds.

2.4. H_2O_2 analysis

H_2O_2 release in the culture medium was measured by chemiluminescence of luminol as described by Glazener et al. [11].

2.5. Enzymatic activity measurements and western blot analysis

Cells (0.5 g) were ground in liquid nitrogen and extracted with 1.5 ml of 200 mM potassium phosphate buffer pH 6 containing 28 mM β-mercaptoethanol, 20 mM sodium metabisulfite, 1% polyclar AT and 0.2 mM PMSF. After centrifugation at 10 000 × *g* for 20 min, the supernatant was desalted over a HiTrap Desalting column (Pharmacia, Sweden) and concentrated using 10 000 Da cut-off microconcentrators (Amicon, USA). UDPG:scopoletin GTase activity was carried out with 40 μl of concentrated extract according to Fraissinet-Tachet et al. [3]. Peroxidase activities towards caffeic and ferulic acids were measured according to [12], those towards scopoletin according to [13]. Scopolin β-glucosidase activity was extracted from BY cells with the above-described buffer supplemented with 1 M NaCl, which was used for solubilization of proteins ionically bound to the cell walls. β-Glucosidase assays were performed at 37°C for 30 min in 100 μl of the reaction mixture containing 50 mM potassium phosphate buffer pH 5, 30 μM scopolin and 50 μl of either desalted enzyme extract or 5-fold concentrated culture medium. Reaction was terminated with 10 μl 1 N HCl and the scopoletin released was analyzed by HPLC. Immunoblotting was performed as described previously [14] and detection was realized with the immun-star chemiluminescent kit (Bio-Rad, France). Polyclonal antibodies raised against recombinant TOGT produced in *Escherichia coli* [3] were used at a 1:10 000 dilution.

3. Results

3.1. Elicitor induction of TOGT accumulation and UDPG:scopoletin GTase activity is not correlated with changes in intracellular scopolin levels

Immunoblot analysis of TOGT protein levels in tobacco cell suspension cultures revealed a band of approximately 50 kDa in non-treated cells, corresponding to the basal level of TOGT (Fig. 1A). Elicitation of tobacco cells with β-megaspermin triggered an early accumulation of TOGT detectable as soon as 1 h after treatment, reaching a maximum at 3 h and

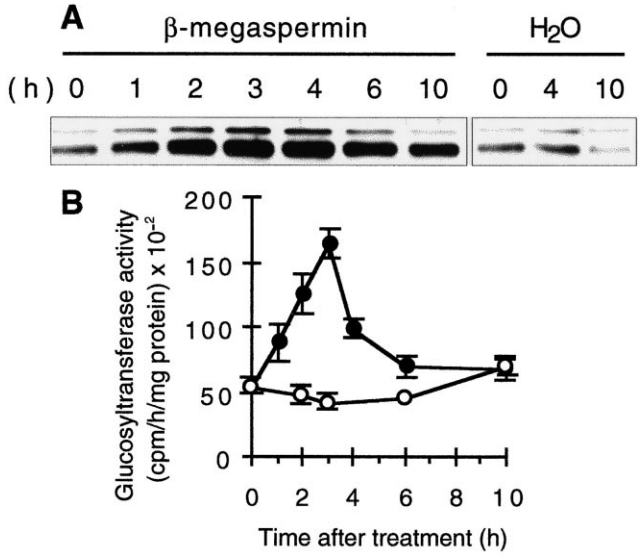

Fig. 1. Western blot analysis of TOGT protein accumulation (A) and induction of UDPG:scopoletin GTase activity (B) in BY tobacco cells treated with 50 nM β-megaspermin (●) or water (○). Standard deviation was calculated from two independent experiments.

then decreasing slowly. Besides the 50 kDa polypeptide, the antiserum also recognized a faint unidentified band of 52 kDa, which might represent another elicitor-inducible TOGT-related protein (Fig. 1A). As scopoletin was the best in vitro substrate of the recombinant TOGT and accumulated in tobacco leaves reacting hypersensitively to TMV [6], time course studies of glucosyltransferase activity were conducted in elicited cells with scopoletin as substrate. UDPG:scopoletin GTase activity of cells treated with β-megaspermin increased transiently, peaking at 3 h and then decreased to the basal level detected in untreated cells (Fig. 1B). These results show a tight correlation between accumulation of TOGT protein and induction of glucosyltransferase activity after elicitation.

The coordinated induction of TOGT accumulation and UDPG:scopoletin GTase activity during elicitation was compared with changes in the levels of the free and conjugated forms of scopoletin. Non-treated tobacco cells contained relatively high amounts (5 nmol/g fresh weight (FW)) of scopolin, the glucosylated form, whereas the levels of the free form were low (∼0.1 nmol/g FW) (Fig. 2). These findings are consistent with the occurrence of preexisting levels of both TOGT and UDPG:scopoletin GTase activity in tobacco cell suspensions. After treatment with β-megaspermin, intracellular scopoletin levels did not change extensively, and there was only a slight increase in elicited cells at 3 h (Fig. 2B). Surprisingly, elicitation induced a sharp decrease in the intracellular scopolin content as soon as 2 h after treatment (Fig. 2A) and only a very low content (∼0.1 nmol/g FW) in the intracellular glucoside was measured at 10 h. These results show that changes in intracellular scopolin levels observed after elicitation did not reflect the induction of TOGT and UDPG:scopoletin GTase activity but rather suggest an enhanced intracellular degradative process or activation of a process of secretion.

3.2. Elicitor treatment induces a rapid secretion and accumulation of scopolin into the extracellular medium

The levels of scopolin and scopoletin present in the culture medium were determined after β-megaspermin treatment. Nei-

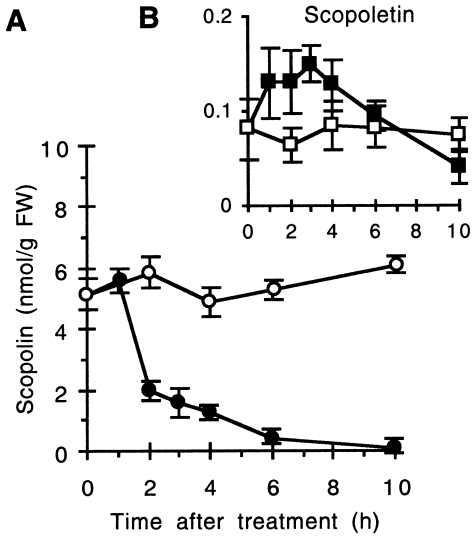

Fig. 2. Levels of (A) intracellular scopolin (●,○) and (B) intracellular scopoletin (■,□) in BY tobacco cells treated with 50 nM β-megaspermin (●,■) or water (○,□). Standard deviation was calculated from two independent experiments.

ther scopolin nor scopoletin could be detected in the culture medium of control cells (Fig. 3A). In contrast, elicitation induced a rapid and massive excretion of scopolin into the extracellular compartment, peaking 3–4 h after treatment (Fig. 3A). Levels of the extracellular glucoside decreased thereafter, suggesting that it was further metabolized. The build-up of scopolin observed in the culture medium was not caused by leakage of the cells because elicitor-induced cell death started only 5 h after β-megaspermin treatment and affected only 30% of the cells after 12 h [15]. No detectable free scopoletin was found in the extracellular medium of elicited cells (Fig. 3A). Acid hydrolysis of the cell walls did not release any scopoletin (data not shown), indicating that it was also not incorporated into cell wall after elicitation. Kinetics of total scopolin accumulation (intracellular plus extracellular scopolin contents) showed that the overall glucoside levels peaked at 3–4 h after elicitation (Fig. 3B). These results are consistent with the induction of TOGT protein and UDPG:scopoletin GTase activity shown in Fig. 1. Overall, our data indicate that during elicitation, the glucosylated form of scopoletin is rapidly synthesized and secreted to the extracellular compartment where it accumulates transiently before being further metabolized.

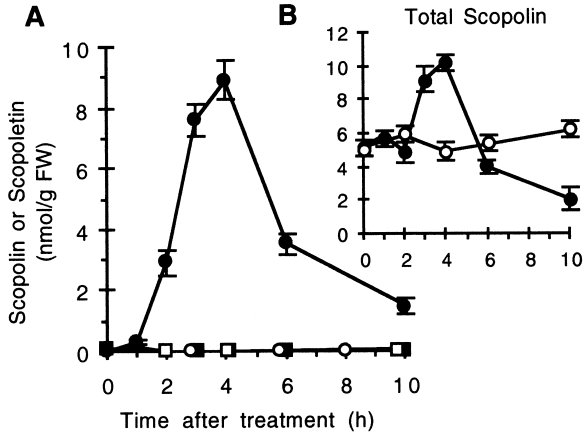

Fig. 3. Levels of extracellular scopolin (●,○) and scopoletin (■,□) in BY tobacco cells treated with 50 nM β-megaspermin (●,■) or water (○,□). A: Extracellular scopolin and scopoletin levels. B: Total (intracellular plus extracellular) scopolin levels. Standard deviation was calculated from two independent experiments.

3.3. *Extracellular scopoletin is metabolized by H_2O_2-dependent oxidation*

It has been previously shown that elicitation of tobacco cells with elicitins resulted in an oxidative burst, probably via activation of an NADPH oxidase complex, and characterized by H_2O_2 production [16] which was recently found to be biphasic upon β-megaspermin treatment [15]. To get further insight into possible relationships between secreted extracellular phenolics and H_2O_2 metabolism, elicited cells were treated with DPI, a suicide inhibitor of the NADPH oxidase in mammalian cells [17]. Exposure of tobacco cells to β-megaspermin resulted in extracellular H_2O_2 accumulation (about 70 nmol/g cells) which was sustained throughout the experiment (Fig. 4A). Treatment of tobacco cells with elicitor and DPI abolished the elicitor-induced H_2O_2 accumulation (Fig. 4A). Changes in intracellular scopoletin and scopolin levels measured in the presence of elicitor and DPI were similar to those measured in the presence of elicitor alone (data not shown). Similarly, DPI had no effect on the elicitor-triggered secretion and extracellular accumulation of the conjugated form scopolin (Fig. 4B). In contrast, when tobacco cells were elicited in the presence of DPI, a strong extracellular

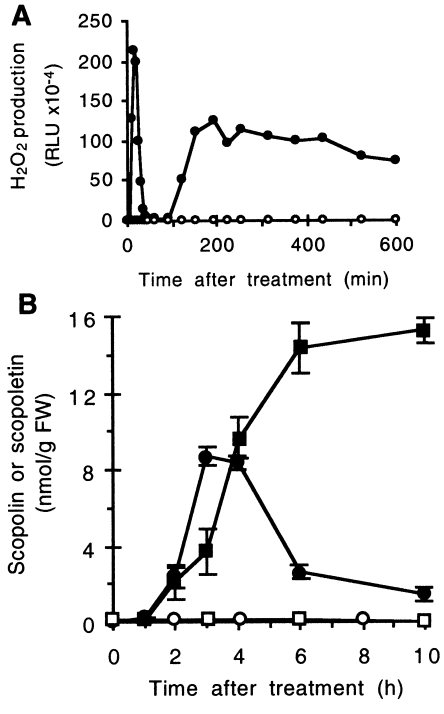

Fig. 4. Relationship between the oxidative burst and scopoletin metabolization. A: H_2O_2 accumulation in the culture medium of BY cells treated with 50 nM β-megaspermin (●) or 50 nM β-megaspermin plus 5 μM DPI (○). RLU: relative luminescence unit. B: Scopolin (●,○) and scopoletin (■,□) accumulation in the culture medium after treatment of BY cells with 50 nM β-megaspermin plus 5 μM DPI (●,■), or DPI alone (○,□). Standard deviation was calculated from two independent experiments.

accumulation of the free form scopoletin occurred (Fig. 4B). The decrease in extracellular scopolin observed from 4 h after elicitation in the presence of DPI matched perfectly the concomitant increase of extracellular scopoletin (Fig. 4B), indicating that scopolin was no longer synthesized and might be hydrolyzed to scopoletin. In support to this, a β-glucosidase activity converting scopolin to scopoletin was detected both in the extracellular medium and in extracts of tobacco cells prepared in presence of 1 M NaCl, indicating that this enzyme is probably apoplastic. Moreover, this activity was induced 2 h after elicitation, peaking at 3 h and then remaining relatively constant throughout the experiment (Fig. 5A), whereas no induction of β-glucosidase activity was observed when it was assayed with *p*-nitrophenyl-β-D-glucopyranoside, a synthetic substrate (data not shown).

As the inhibition of H_2O_2 formation by DPI resulted in extracellular scopoletin accumulation after elicitation and as scopoletin is a known peroxidase substrate in vitro [8], peroxidase activity towards scopoletin was analyzed in elicited cell suspension cultures. Crude enzyme extracts from tobacco cells and culture medium were shown to immediately catalyze the oxidation of scopoletin in the presence of H_2O_2 (Fig. 5B). Scopoletin was not oxidized in the absence of either H_2O_2 or enzyme extract indicating that it is enzymatically metabolized by peroxidases. Scopoletin peroxidase activity was constitutively present in tobacco cells and culture medium and did not increase upon elicitation (Fig. 5B). Together, these results show that after elicitation of tobacco cell suspension cultures, secreted scopolin might be cleaved into the free form, which is immediately metabolized in the presence of constitutive peroxidases and of elicitor-induced H_2O_2.

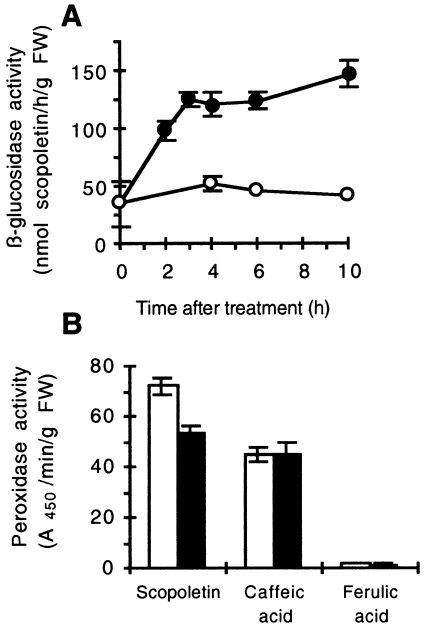

Fig. 5. β-Glucosidase and peroxidase activities. The values are the sum of the activities in cell extract and in culture medium. A: Scopolin β-glucosidase activity after treatment with 50 nM β-megaspermin (●) or water (○). B: Peroxidase activity towards scopoletin, caffeic and ferulic acid ($A_{450\,nm}$) was measured 4 h after treatment with either 50 nM β-megaspermin (black bars) or water (white bars). Standard deviation was calculated from two independent experiments.

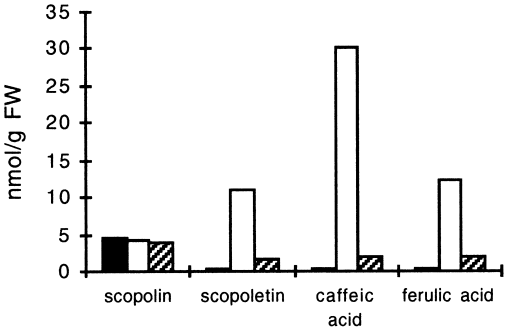

Fig. 6. Effect of DPI and exogenous H_2O_2 on extracellular accumulation of scopolin, scopoletin, caffeic acid and ferulic acid. Tobacco cells were treated with 50 nM β-megaspermin (black bars), 50 nM β-megaspermin plus 5 μM DPI (white bars), or 50 nM β-megaspermin plus 5 μM DPI plus 80 μM H_2O_2 (hatched bars). Culture medium was harvested 4 h after treatment with β-megaspermin. H_2O_2 (80 μM final concentration) was added to DPI-treated cells 30 min before harvest.

3.4. Secreted hydroxycinnamic acids might also act as H_2O_2 scavengers

When tobacco cells were elicited in the presence of DPI, two additional low molecular weight phenolics identified as free caffeic and ferulic acids, respectively, accumulated extracellularly (Fig. 6). No accumulation of these hydroxycinnamic acids was observed in the culture medium of elicited cells without DPI or in control cells. To investigate whether caffeic and ferulic acids could be esterified in the cell walls, the cell wall fraction was treated with alkali. However, this treatment did not release any caffeic or ferulic acids (data not shown). Both compounds were also substrates of constitutive peroxidases (Fig. 5B). However, peroxidase activity towards ferulic acid, although significant, proved to be much lower than when assayed with scopoletin or caffeic acid. Cells elicited in the presence of DPI were treated with exogenous H_2O_2 added at concentrations mimicking those found in the extracellular medium of elicited cells without DPI. As shown in Fig. 6, addition of H_2O_2 (80 μM) significantly reduced the level of scopoletin, caffeic and ferulic acids found in the extracellular medium of elicited cells in the presence of DPI, showing that H_2O_2 is necessary for further metabolization of these free phenolics. Unlike scopoletin, extracellular scopolin accumulation was not affected by the addition of H_2O_2 (Fig. 6) indicating that the glucoside was not a substrate of peroxidases.

4. Discussion

Our results show that elicitation of tobacco cells triggers a rapid synthesis and secretion of the hydroxycoumarin glucoside scopolin. The coordinated induction of TOGT accumulation and UDPG-scopoletin:GTase activity upon elicitation strongly suggests the involvement of TOGT in the conjugation reaction leading to scopolin formation. Here, scopolin was shown to represent a transportable form of the hydroxycoumarin scopoletin, its highly reactive phenolic hydroxyl being protected through O-glucosylation against cellular oxidases. Formation of soluble glucosides is generally assumed to be a prerequisite for transport of phenolics to the apoplasm. For example, in cell suspension cultures of *Chenopodium rubrum*, feruloylglucose, along with 4-coumaroylglucose might represent important intermediates that would be discharged in

the apoplastic space prior to their incorporation into the cell wall [18]. Likewise, during lignin formation, glucosylation of monolignol precursors is thought to be necessary for their transport to the cell wall [2]. In plant cells, glucosides and the corresponding reactive aglycone-releasing β-glucosidases are generally located in different cellular compartments [19]. In sweet clover leaves, glucosides of 2-hydroxycinnamic acid are present in the vacuoles of mesophyll cells, whereas most of the β-glucosidase is located in the extracytoplasmic space [20]. In our model system, β-megaspermin elicitation induced secretion of the intracellularly produced scopolin, allowing its cleavage by extracellular β-glucosidases and subsequent release of the reactive form scopoletin. These findings support the idea that glycosylation may serve to provide a pool of inactive and transportable forms of compounds that can be rapidly obtained in active forms by specific β-glucosidases, as observed for cytokinin conjugates in roots of maize [21] and for salicylic acid β-*O*-D-glucoside in tobacco [22].

We have shown that after induction of defense responses, extracellular free scopoletin, as well as secreted caffeic and ferulic acids were rapidly metabolized via a peroxidative reaction involving H_2O_2 consumption. Our data clearly indicate that these abundant plant compounds represent potent antioxidants in vivo that can act in a similar manner to ascorbate or glutathione [23] and function as electron donors for the peroxidase reaction. Besides the well-recognized role of phenylpropanoids as antimicrobial agents and precursors for cell wall impregnation in plants, the potent antioxidant properties of dietary flavonoids and hydroxycinnamic acids have also recently received a great deal of attention [9]. Our results show that free phenylpropanoids, along with peroxidases, could have a similar function in plants and act as direct scavengers of H_2O_2 produced after induction of defense responses. These data are in accordance with recent work suggesting that phenolic acids could buffer the damaging effects of ROIs occurring during senescence [24]. Excess H_2O_2 has actually been shown to provoke cell death in catalase deficient tobacco [25], and *Arabidopsis* mutants defective in flavonoids and sinapate esters are also more sensitive to oxidative damage caused by UV-B [26]. As H_2O_2 is considered as a diffusible signal for plant defense reactions [27], free apoplastic phenolics may be involved in the control of the spread of signalling ROIs during the HR.

Metabolites such as glutathione and ascorbate, as well as antioxidant enzymes are known to be important determinants of resistance of plants to oxidative stresses [28]. Together, our data suggest that additional protection mechanisms may exist and that the major function of the TOGT could be its involvement in a mechanism of induced secretion of potent antioxidant phenylpropanoids in tobacco, thus participating in the control of the cell's redox state during plant-pathogen interactions.

Acknowledgements: The authors are grateful to S. Kauffmann for providing the β-megaspermin. This work was supported by a postdoctoral Rhône-Poulenc fellowship to R.B. and by Grant 97-5-11603 from the French Ministry of Research to J.C.

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