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Scopoletin Production Related to Induced Resistance of Tobacco Plants Against *Tobacco mosaic virus*

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A fluorescent material was accumulated in inoculated leaves showing necrotic local lesions of tobacco plants with N gene, *Nicotiana tabacum* cvs. Xanthi-nc NN, Samsun NN, Burley 21 and KF 114, and *N. glutinosa*, and *Datura stramonium* at the early growth stages by the inoculation of *Tobacco mosaic virus* (TMV). It was identified as a coumarin phytoalexin, scopoletin. Although the material was most prominently produced in TMV-inoculated tobacco leaves with local necrotic lesions, its accumulation was also noted in uninoculated leaves of TMV-inoculated plants. Its accumulation was somewhat greater in high resistance-induced leaves than low resistance-induced and intact leaves. Scopoletin treatment induced the expression of a pathogenesis-related protein, PR-1, prominently at the concentrations of 500 or 1000 μg/ml. This suggests that scopoletin is a phytoalexin abundantly accumulating in N gene-containing resistant plants in response to TMV infection, and may be related to hypersensitive responses (HR) and systemic acquired resistance (SAR) in the resistant tobacco plants.

Keywords : pathogenesis-related protein, scopoletin, systemic acquired resistance, tobacco, *Tobacco mosaic virus*.

Phytoalexins are produced and accumulated in plants that come in contact with pathogens or by chemical or physical stimuli. They are synthesized by the activation of phenylpropanoid biosynthesis pathway and regarded as general defense chemicals against various microbial pathogens (Dixon, 1995; Kuć, 1997; Smith, 1996). Especially, phytoalexins are produced more specifically or prominently in incompatible reactions of plants to pathogen infection, usually accompanying hypersensitive responses (HR) characterized by rapid death of infected cells (localized necrosis). In the incompatible reactions, localized cell death in HR can block the further growth and spread of viruses and biotrophic microbes, but not for facultative parasites which colonize dying or dead tissues (Strange, 1998). One of other defense responses to these facultative parasites is synthesis and accumulation of phytoalexins which are generally fungitoxic and antibacterial (Smith, 1982), and functioning as a chemical barrier.

After HR and localized necrosis, plants may become more resistant to subsequent pathogen attack. This is known to be systemic acquired resistance (SAR) or induced resistance (Ross, 1961; Sticher et al., 1997). Salicylic acid is a main compound that plays a role as a signal molecule for SAR, accompanying the expression of pathogenesis-related (PR) proteins (Antoniw et al., 1980; Yalpani et al., 1991). Phytoalexin accumulation is also related to one of mechanisms in SAR. However, the specific role of phytoalexins in disease resistance and SAR is not clear for the majority of host-parasite systems, and there are few reports showing that they provide SAR to infection. Research on phytoalexins has mainly relied on the identification of induced antimicrobial compounds and correlating their presence with resistance (Hammerschmidt and Dann, 1999).

In case of the tobacco - *Tobacco mosaic virus* (TMV) interaction, which has been used as a model system for SAR or induced resistance, various phenolic phytoalexins are identified, including chlorogenic acid, 4- and 5-caffeoyl acids, 3-, 4-, and 5-feruloylquinic acids, 3-*p*-coumaryl quinic acid, 1-caffeoyl, feruloyl and *o*-coumaroyl esters of glucose, 1-*o*-coumaryl gentiobiose, scopolin, and rutin (Tanguy and Martin, 1972). However, they have rarely been specifically related to SAR or seldom compared for their functions in SAR among phytoalexins and examined for which one is involved more in SAR. Therefore, we examined allelochemicals specifically produced in hypersensitive responses of tobacco plants with N gene to TMV infection, and tested the relationships with the induction of resistance in tobacco plants.

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Materials and Methods

Detection of specific materials produced in resistant tobacco plants infected with TMV. A local lesion host of TMV, *Nicotiana tabacum* cv. Xanthi-nc NN, and a susceptible systemic host *N. tabacum* cv. NC 82, both at 6-8 leaf stages, were mechanically inoculated with TMV common strain by rubbing leaf surface with cotton swab after dusting 500-mesh carborundum, and placed in a greenhouse at 25 ± 2°C. Inoculated Xanthi-nc NN leaves showed necrotic lesions from 2-3 days after inoculation, and in NC 82, mosaic symptoms appeared on the upper leaves approximately one week after inoculation. Infected leaves were harvested (after 5 days for Xanthi-nc NN and after 2 weeks for NC 82), macerated in liquid nitrogen, and extracted with ethanol. The ethanol extract was centrifuged at 20,000 g, and supernatant was evaporated *in vacuo* for dryness. The ethanol extract was dissolved in chloroform and spotted on thin layer chromatography (TLC) plates and developed in a chloroform : methanol = 30 : 1 solvent system or other various solvent systems. Results of TLC were compared among healthy and diseased Xanthi-nc NN and diseased NC 82.

Purification and identification of a fluorescent material specifically produced in resistant plants. The dried ethanol extract of the infected tobacco leaves was separated in a separation funnel using equal volumes of *n*-hexane and methanol. The fluorescent material was separated into the methanol layer, which was further isolated and purified using preparative TLC (Silica gel RP-18 F_{254S}, Merck, Darmstadt, Germany) and HPLC on a 20 mm i.d. × 250 mm Nucleosil C18, 7 μm column in 70% aqueous acetonitrile, respectively. The purified material was dissolved in chloroform and analyzed by using nuclear magnetic resonance (NMR).

Induced systemic resistance related to the production of the fluorescent material. Several tobacco cultivars with N gene were inoculated with TMV. Necrotic lesions were formed on the inoculated leaves from 3 days after inoculation. After 15-20 days, non-inoculated fresh upper leaves of the plants were used for inoculation of TMV and isolation of the fluorescent material. Briefly, the right or left side from mid vein of a leaf was inoculated with TMV as above, and lesion formation was examined. The other half side of the leaf was cut off and macerated in liquid nitrogen, which was extracted in ethanol and separated by TLC as above to visualize the fluorescent material. Leaves with necrotic lesions formed by the 1st inoculation, and healthy leaves were examined to compare the presence and approximate amount of the fluorescent material.

PR gene induction by the treatment of the fluorescent material. The purified fluorescent material (500 μg/ml) was dissolved in 30% acetone and treated on Xanthi-nc NN leaves. Twenty-four hours after treatment, total RNA was isolated from frozen leaf tissues by phenol/chloroform extraction followed by LiCl precipitation (Choi et al., 1992). About 20 μg samples of total RNA were separated by electrophoresis through formaldehyde agarose gels. After electrophoresis, the electrophoresed gel was blotted to nylon membrane overnight and probed by the ^{32}P-dCTP labeled PR-1 cDNA probe. Hybridization was carried out in 50% formamide at 42°C. Tobacco leaves treated with 30% acetone alone, another fraction on TLC specifically detected in TMV-infected Xanthi-nc NN leaves with local necrotic lesions, an SAR-inducing material (benzothiadiazole, BTH), and salicylic acid were analyzed together for examining the PR gene expression.

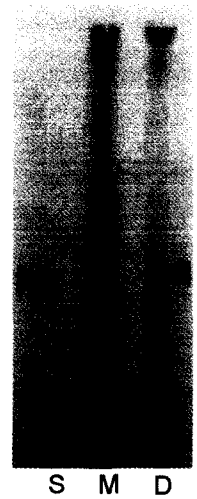

Fig. 1. Thin layer chromatography of ethanol extracts of tobacco leaves (chloroform : methanol = 30 : 1). S: purified fluorescent material (scopoletin), M: NC 82 leaves showing systemic mosaic symptoms by the infection of TMV-common strain, D: Xanthi-nc NN leaves with local lesions by TMV infection. Arrow heads indicate the same fluorescent material (*Rf* value of 0.4).

Results

Production and identification of scopoletin in resistant tobacco plants infected with TMV. When the ethanol extracts of Xanthi-nc NN leaves with local lesions formed by TMV infection, healthy Xanthi-nc NN leaves, NC 82 leaves with systemic mosaic symptoms were compared by TLC, a fluorescent material under long-wave length UV (365 nm) with *Rf* value of 0.4 was detected prominently only in the diseased Xanthi-nc NN tobacco (Fig. 1). This material was not detected in healthy tobacco leaves and NC tobacco leaves with systemic mosaic symptoms at the early stage of growth. In other experiments, this material was also produced prominently in local lesion host plants such as *Datura stramonium, N. glutinosa, N. tabacum* cvs. Samsun NN, KF 114 (a commercial cultivar with N gene), and Burely 21 (data not shown).

With proton NMR analysis, the fluorescent material was identical to scopoletin (6-methoxy-7 hydroxycoumarin, Aldrich 24,658-1), and was confirmed by comparing it with commercial scopoletin by TLC (data not shown).

Induction of resistance related to scopoletin production. Four days after TMV inoculation on the upper leaves of inoculated leaves, local lesions were measured in size. The lesion sizes varied depending on tobacco cultivars, plants, and leaves in the same plant, ranging from 0 to 121% rela-

Table 1. Induction of resistance by pre-inoculation of TMV on resistant tobacco plants

Cultivar	Leaf No.	Size of local lesion[a] (diameter, mm)	% lesion area relative to control
Xanthi-nc	X(Control)[b]	2.36[c]	100
	XA - 1	1.76	74.5
	XA - 2	1.21	51.3
	XB - 1	2.04	86.4
	XB - 2	2.12	89.8
KF 114	F (Control)	1.52	100
	FA - 1	0.49	32.2
	FA - 2	0.00	0.0
	FB - 1	1.84	121.1
	FB - 2	1.41	92.7
Samsun NN	S (Control)	1.74	100
	SA - 1	1.43	82.2
	SA - 2	1.11	63.8
	SB - 1	1.17	67.2
	SB - 2	1.00	57.5
	SC - 1	1.35	77.6
	SC - 2	0.00	0.0
	SD - 1	1.71	98.3
	SD - 2	1.74	100.0
Nicotiana glutinosa	N (Control)	2.30	100
	NA - 1	1.74	75.7
	NA - 2	1.62	70.4

[a] Upper half leaves were inoculated by TMV 15-20 days after preinoculation of the lower leaves, and lesion size was measured 4 days after inoculation.
[b] Control: plants of the same growth stage without preinoculation.
[c] Average of 10 replications.

tive to those of control leaves that were inoculated for the first time, but generally having smaller lesion sizes (Table 1).

Of the 18 leaf samples examined for induced systemic resistance, 7 samples with high or low resistance were selected and scopoletin production was examined by TLC. The leaf with necrotic lesions (Samsun NN) had the most prominent production of scopoletin as revealed by TLC, and the fluorescent material was detected strongly under UV of 365 nm for KF 114 FA and Samsun NN SC-2 leaves which had high induced resistance (Fig. 2). No or little of the fluorescent material was detected in the leaves with low resistant level and control leaves.

Induction of PR-1 gene expression by scopoletin treatment. A strong expression of *PR-1* gene was detected when the fluorescent material (scopoletin) from TMV-infected necrotic leaves was treated on tobacco leaves, while treatment of an unknown material (detected at the *Rf* value of 0.1 with chloroform : methanol = 30 : 1 by short wavelength UV, 254 nm, in TLC) specifically formed on Xanthi-nc NN by TMV infection and the control revealed no

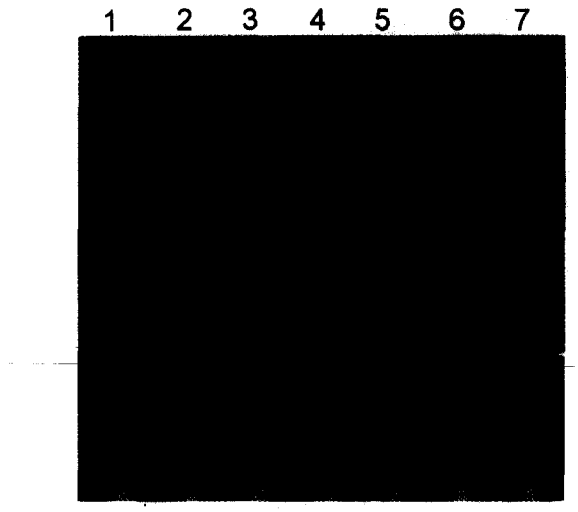

Fig. 2. Thin layer chromatography of ethanol extracts of tobacco leaves with resistance induced by pre-inoculation of lower leaves. Solvent system of TLC; *n*-hexane: toluene: diethyl ether: methanol: acetic acid = 50 : 25 : 15 : 5 : 5. Lane 1: healthy KF 114 (F), Lane 2: resistance-induced KF 114 (mixture of FB-1 and FB-2), Lane 3: resistance-induced Samsun NN (mixture of SD-1 and SD-2), Lane 4: resistance-induced Samsun NN (SC-1), Lane 5: resistance-induced KF 114 (mixture of FA-1 and FA-2), Lane 6: resistance-induced Samsun NN (SC-2), Lane 7: Samsun NN with necrotic local lesions (cf. Table 1).

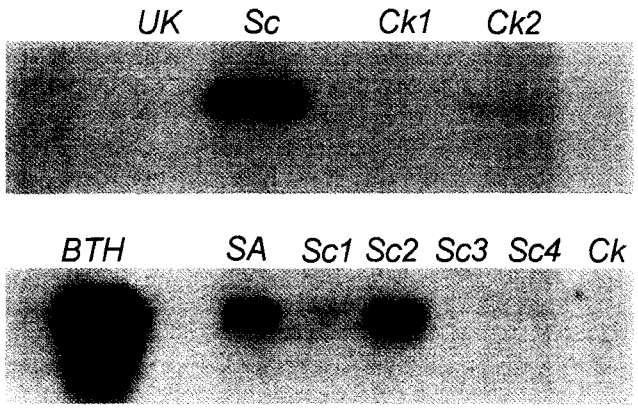

Fig. 3. Northern blot analysis of *PR-1* gene expression induced in Xanthi-nc NN by the treatment of purified fluorescent material (scopoletin, Sc) (500 μg/ml) (upper) and commercial scopoletin (Sc) and other chemicals (lower). RNA was extracted from inoculated leaves 24 hours after treatment. **Upper**: Cks 1 and 2; different plants treated with acetone alone, Uk; unknown material isolated from TMV-infected Xanthi-nc NN leaves. **Lower**: Ck; control, BTH; benzothiadiazole, SA; salicylic acid (1 mM), Sc1; 2,000 μg/ml, Sc2; 1,000 μg/ml, Sc3; 500 μg/ml, SC4; 250 μg/ml of commercial scopoletin.

induction of *PR-1* gene (Fig. 3). In different levels of scopoletin treatment, PR-1 gene was strongly expressed at 1,000 μg/ml, similar to 1 mM salicylic acid, however, the gene expression was reduced at 2,000 μg/ml and not

detected at the lower concentrations (Fig. 3). Benzothiadiazole treatment always induced the strongest *PR-1* gene expression in several repeated experiments.

Discussion

Scopoletin, a simple 7-hydroxylated coumarin and a normal phenolic constituent present in several plant species, is known to be produced and accumulate in soloanaceous plants upon pathogen infection and generally considered to be an antimicrobial compound (Kuc, 1982). In tobacco, high levels of scopoletin accumulated in stunted plants by the infection of *Peronospora hyoscyami* f. sp. *tabacina* (the incitant of blue mold) (Reuveni and Cohen, 1978). Scopolin, glucoside of scopoletin, is listed as one of phytoalexins accumulated by TMV infection (Tanguy and Martin, 1972). Recently, it was reported that scopoletin accumulation was observed in cultured tobacco cells in response to fungal elicitor, and also responsible for the fluorescent rings observed around local lesions occurring in HR to TMV (Schalk et al., 1998). Like salicylic acid (SA), which was shown to accumulate in plants with HR and to be essential for local resistance and SAR (Gaffney et al., 1993; Delaney et al., 1994), scopoletin is produced via the phenylpropanoid pathway involving the first committed enzyme of this pathway and a major defense-related and resistance-inducing enzyme, phenylalanine ammonia lyase (PAL) (Mauch-Mani and Slusarenko, 1996).

Scopoletin may be a normal constituent of mature tobacco leaves which is transferred to smoke as fluorescing particulate matter (Romert et al., 1994; Jenkins et al., 1996) and is produced in the tissue culture (Reinhard et al., 1967). When leaf extracts of young tobacco plants were analyzed by TLC in our study, detectable amounts of scopoletin seemed not to accumulate in healthy plants and susceptible plants with mosaic symptoms induced by TMV infection. It accumulated rather specifically in Xanthi-nc NN tobacco leaves with necrotic local lesions due to TMV infection at the early growth stage. Its accumulation was also noted in the inoculated leaves of other resistant tobacco plants *D. stramonium* in our study, suggesting that it may be a common phenomenon related to HR against TMV infection.

It is well known that following the HR, plants may become more resistant to subsequent pathogen attack, a phenomenon called SAR (Ross, 1961), and that in conjunction with the HR and SAR, PR proteins are expressed locally and systemically by virus infection, respectively (Antoniw et al., 1980, Bol et al., 1990; Kim et al., 1996). Defense reactions related to the HR involve the stimulation of PAL (Hahlbrock and Scheel, 1989; Dorey et al., 1997) which is involved in biosynthesis of SA and scopoletin (Mauch-Mani and Slusarenko, 1996; Fritig et al., 1970). SA is regarded as a signal molecule for HR and SAR (Silverman et al., 1993), expressing PR genes, especially *PR-1* (Malamy et al., 1990; Malamy and Klessig, 1992). In our study, scopoletin accumulated more in higher resistant tobacco leaves than lower or no resistant ones. This result is not definitely showing that scopoletin is directly related to SAR since TMV-induced lesion size decreases by about 60% (Kim et al., 1998) and the endogenous SA levels increase 10-fold in uninoculated leaves of the TMV-inoculated Xanthi-nc NN tobacco plants (Malamy et al., 1990). Scopoletin accumulation in resistant tobacco plants, however, may be served as an indication of increased levels of resistance against TMV infection probably by the suppression of viral replication and thereby restriction of lesion size because the intercellular fluid of hypersensitive tobacco infected with TMV contains the inhibitor of virus replication (Spiegel et al., 1989).

Until now the expression of PR genes has not been documented well in relation to scopoletin accumulation. There have been few attempts to determine if scopoletin suppresses viral replication and induces PR gene expression. In our study, the exogenous treatment decreased somewhat the lesion sizes produced by TMV infection on Xanthi-nc NN tobacco leaves (data not shown). Infiltration of scopoletin into tobacco leaves readily induced *PR-1* gene expression. The induction of PR-1 gene expression through scopoletin treatment is now equivocal whether it acts on the phenylpropanoid pathway or other pathways. Also the *PR-1* gene expression was not governed in a dosage-dependent manner, showing the complexity of its activity. Therefore, more study is needed to explain the role of scopoletin in the induction of local and systemic resistance, and PR proteins.

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