Morning Glory Systemically Accumulates Scopoletin and Scopolin after Interaction with *Fusarium oxysporum*

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An isolate of non-pathogenic *Fusarium*, *Fusarium oxysporum* 101-2 (NPF), induces resistance in the cuttings of morning glory against *Fusarium* wilt caused by *F. oxysporum* f. sp. *batatas* O-17 (PF). The effect of NPF on phenylpropanoid metabolism in morning glory cuttings was studied. It was found that morning glory tissues responded to treatment with NPF bud-cell suspension (108 bud-cells/ml) with the activation of phenylalanine ammonia-lyase (PAL). PAL activity was induced faster and greater in the NPF-treated cuttings compared to cuttings of a distilled water control. High performance liquid chromatography analysis of the extract from tissues of morning glory cuttings after NPF treatment showed a quicker induction of scopoletin and scopolin synthesis than that seen in the control cuttings. PF also the induced synthesis of these compounds at 10^5 bud-cells/ml, but inhibited it at 10^8 bud-cells/ml. Possibly PF produced constituent(s) that elicited the inhibitory effect on induction of the resistance reaction. These compounds could potentially be useful as markers to detect early beginning interactions between *Fusarium* and morning glory tissues cuttings.

Key words: Morning Glory, Induced Resistance, Phenylpropanoid

Introduction

In recent years, biological control of plant diseases, using non- or weakly pathogenic microorganisms, has attracted considerable attention as an environmentally sensitive method of crop protection. Utilization of non-pathogenic *Fusarium* (NPF) is one of the most successful examples and has been shown to be effective in the control of wilt disease of spinach (Katsube and Akasaka, 1997), tomato (Watanabe *et al.*, 1991) and sweet potato (Ogawa and Komada, 1984; Ogawa, 1988). Although the underlying mechanism for the expression of the controlling effect is extremely important for the development of new types of pest-management, it has not been yet well understood. However, it is very likely that it is related to the defense potential of plants.

After coming into contact with pathogenic microbes, plants are usually capable of recognizing the pathogen and subsequently activate diversified defense responses in a concerted manner to prevent infection. Current data from successful examples of biological control using NPF suggest that the pretreatment of a plant with a non-pathogen induces no defense response itself, but instead induces a 'primed' state, in which the plant is ready to rapidly fight against pathogen infection (Ogawa, 1988). The mechanism of this 'primed' state is in the center of interest.

Our previous work showed that treatment with NPF induced resistance against pathogenic *Fusarium* in morning glory seedlings (Shimizu *et al.*, 2000). Due to the ease of the plant material handling, this system represents a facile laboratory model for the study of interactions between NPF and the plant. Resistance was induced by the supernatant of the NPF bud-cell suspension, strongly suggesting that the inducers are a chemical factor(s). Using the model system, it has been demonstrated that the acquisition of the resistance by the plant occurrs after 6–10 h of interaction with NPF, although the protective effect is not evident until about 7 d after the *F. oxysporum* f. sp. *batatas* (PF) inoculation. This rapid induction of resistance in morning glory suggests that early reactions play important roles in establishing resistance.

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In this study, the reaction of morning glory following treatment with NPF was examined to find biochemical marker(s) that would allow an easier assessment of the induction of resistance against PF. This project specifically focused on the change in composition of secondary metabolites, since secondary metabolism has been shown to be related to plant defense in many cases.

Materials and Methods

Fungal and chemical materials

Isolates of *Fusarium oxysporum* 101-2 (non-pathogenic; NPF) and *F. oxysporum* f. sp. *batatas* O-17 (pathogenic; PF), maintained in potato-sucrose-agar medium, were used. They were cultured by shaking in potato dextrose broth (DIFCO) for 7 dat 28 °C. The culture was then filtered through a layer of gauze and centrifuged at 3,000 × *g* for 5 min. The pellet of bud-cells was suspended in distilled water and used for the plant treatment after adjusting the bud-cell concentration. The cell-free NPF culture solution was prepared by centrifuging the suspension of NPF bud-cells at 15,000 × *g* for 20 min. The fungal culture and the cell-free culture solution were autoclaved at 120 °C for 15 min to give a dead fungal culture.

Scopoletin was purchased from Fluka. Scopolin was isolated from the treated morning glory cuttings as described below.

Plant material and treatment

The seeds of morning glory (*Ipomoea tricolor* cv. heavenly blue), purchased from Sakata Seed Co. (Yokohama, Japan), were sown on vermiculite and grown under continuous light at 25 °C, and watered with 0.1% (v/v) HYPONeX® (HYPONeX Japan, Osaka) as a fertilizer solution. A stem of ~ 5 cm length with cotyledons was excised from 7-day-old seedlings, and the cuttings were put into NPF bud-cell suspension, the supernatant of the culture of NPF, or in an aqueous solution of the test chemicals. The solution of chemicals contained 1% (v/v) ethanol, as did all controls. After a designated period, the cuttings were transferred to a PF bud-cell suspension containing 0.05% (v/v) HYPONeX® and kept at 25 °C. Distilled water containing 0.05% (v/v) HYPONeX® was used for the control treatment.

Assay of phenylalanine ammonia-lyase (PAL) activity

The treated cuttings were cut into portions: cotyledons (~ 0.16 g fresh weight, FW), upper and lower half of stem (~ 0.13 g fresh weight, 2.5 cm each). The plant samples were then ground in a mortar with sea sand in the presence of 1 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.4) containing 0.1% (v/v) 2-mercaptoethanol on ice, followed by centrifugation at 15,000 × *g* for 10 min. The supernatant (100 µl) was added into 100 µl of the reaction buffer (100 mM Tris-HCl, pH 8.4, containing 10 mM L-phenylalanine) and kept at 30 °C for 30 min. The reaction was stopped by adding 50 µl of acetic acid. An aliquot (20 µl) of the reaction solution was analyzed by reversed phase HPLC using a C-18 column (COSMOSIL 5C18-AR II, 150 × 4.6 mm I.D., Nacalai Tesque, Kyoto, Japan) and eluted with 60% (v/v) aqueous methanol containing 0.1% (v/v) formic acid. The amount of the product, *trans*-cinnamate, was quantified on the basis of the peak area detected at 280 nm. The protein content in the supernatant was determined by a Bio-Rad® protein assay kit using bovine serum albumin as a standard. Phenylalanine ammonia-lyase (PAL) activity was expressed by katal (kat) per µg protein assayed.

Isolation and structure elucidation of scopolin

The cuttings were treated with NPF (10^8 bud-cells/ml) for 20 h and subsequently with sterilized water for 28 h. The stem part of the cuttings was collected (120 g) and homogenized in 80% (v/v) aqueous methanol (300 ml) with a mechanical homogenizer (Nihonseiki). After centrifugation at 3,000 × *g* for 10 min, the supernatant was evaporated under reduced pressure, and the residue was dissolved in 50% (v/v) aqueous methanol (2 ml) and applied to a Sep-Pak® C18 cartridge (Waters) equilibrated with water. The cartridge was eluted with 50% (v/v) aqueous methanol (5 ml), and the eluate was subjected to reversed phase HPLC separation using a COSMOSIL 5C18-AR column (250 × 20 mm I.D.) and a mobile phase of 15% (v/v) aqueous methanol containing 0.1% (v/v) formic acid at a flow rate of 7.0 ml/min. The elution was monitored by UV absorption at 254 nm. The total yield of scopolin, 5.3 mg, was identified by spectroscopic analysis. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-300 instrument.

The mass spectrum was obtained with a Perkin-Elmer Sciex API 165 operating in electrospray ionization mode (positive mode, 70 eV, methanol solution of the sample was introduced by a continuous flow at 5 μl/min). For the identification of the sugar moiety of the obtained scopolin, the sample (3 mg) was hydrolyzed in 1 N hydrochloric acid (2 ml) at 85 °C for 2 h. The reaction solution was then evaporated under reduced pressure and the residue was dissolved in water (1 ml) and passed through a Sep-Pak® C18 cartridge equilibrated with 0.1% (v/v) formic acid. The cartridge was washed with 0.1% (v/v) aqueous formic acid (6 ml), and the combined eluates were dried under reduced pressure. The residue was dissolved in the reaction reagents mixture (1 ml; dry pyridine/trimethylchlorosilane/hexamethyldisilazane 10:1:2, v/v/v), and allowed to stand for 15 min. An aliquot (1 μl) of these samples was analyzed by a gas chromatograph (G-5000, Hitachi) equipped with a Quadrex® MS capillary column (methyl silicon, 25 m × 0.25 mm I.D., 0.25 μm film thickness) and a flame ionization detector. Helium was used as the carrier gas with a linear velocity of 28.1 cm/min. The injection port and detection port were kept at 180 °C and the oven temperature was raised from 160 to 180 °C at 1 °C/min with a final hold at 180 °C.

Quantification of scopolin and scopoletin

The lower part of the stem (2.5 cm) from two cuttings was extracted by heating in 2 ml of 10% (v/v) aqueous acetonitrile containing 2% (v/v) acetic acid for 10 min. The extracts were then centrifuged at 3,000 × *g*, and the obtained supernatants were subjected to HPLC analysis using a YMC-Pak ODS-A (150 × 4.6 mm I.D.) column and a solvent mixture of methanol/water containing 0.1% (v/v) aqueous formic acid at a flow rate of 0.8 ml/min. The solvent gradient was programmed as follows: 0–2 min, 1:4 (v/v); 2–30 min, linear increase from 1:4 to 4:1 (v/v). The peak was detected by fluorescence at 420 nm (excitation at 340 nm).

Antifungal activity

For germination inhibition assays, PF bud-cells (10^5 bud-cells/ml) were suspended in scopoletin (100, 10 μg/ml) or scopolin (100, 10 μg/ml) solution containing 1% (v/v) ethanol and 1% (w/v) sucrose. After incubation at 25 °C for 24 h with gentle shaking in the dark, the rate of germination was determined by microscopic observation.

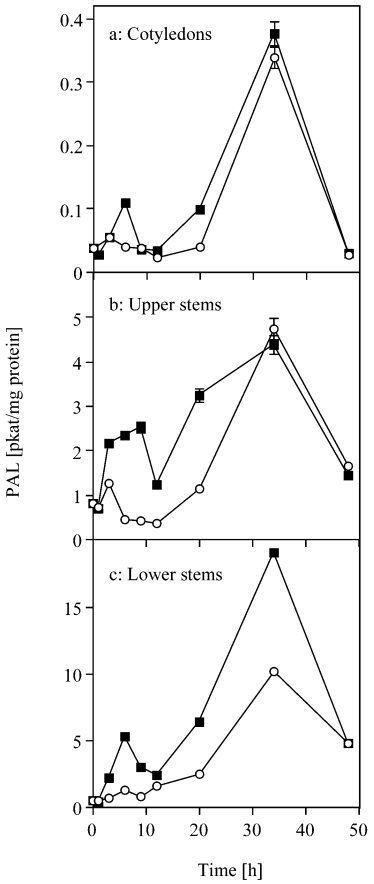

Fig. 1. Changes in PAL activity in the cuttings of morning glory, control (○) or NPF-treated (■). Treatments were started at 0 h and the cuttings were transferred to distilled water after 20 h. NPF was given at 10^8 bud-cells/ml. The cuttings were excised into three portions, cotyledons (a), upper half (b) and lower half (c) of stems. The data show the average of the PAL activity per protein assayed. The unit kat means katal defined as the activity of enzymes forming 1 mol of *trans*-cinnamic acid from the substrate phenylalanine per second. The horizontal axis shows the period of treatment. Bars show standard errors (n = 3).

Effect of scopoletin and scopolin on susceptibility to PF

Morning glory cuttings were placed into an aqueous solution of scopoletin or scopolin containing 1% (v/v) ethanol and kept at 25 °C for 20 h under light conditions. The cuttings were subsequently transplanted into PF suspension (10^5

bud-cells/ml) containing 0.05% (v/v) HYPONeX® and kept for 7 d at 25 °C under light conditions. The symptoms induced on the cotyledons of treated cuttings were evaluated by quantifying the amount of chlorophyll according to previously reported methods (Shimizu *et al.*, 2000).

Results

Changes in PAL activity in morning glory cuttings

The treatment of morning glory cuttings with NPF caused an increase in the PAL activity in all parts of the cuttings. The PAL levels started to rise at 3 h post treatment. The level in control cuttings has also increased, although the increase was evident after 12 h (Fig. 1). Among the parts of the cuttings, the largest level and increase were observed in the lower stem.

In all the parts of the cuttings treated with NPF, the increase occurred in two phases, the first and second peaks were seen after 6 h and 34 h, respectively. The second peak was higher than the first one: 3.5-fold in the lower stem. The control cuttings showed a single increase only after 34 h.

At the first phase, there were significant differences of the PAL level between NPF-treated and control cuttings in all the parts. In the lower stem, the level in NPF-treated cuttings was 3 times higher than that in the control cuttings. There was no significant difference in the second phase of the upper stem and cotyledons, although NPF treatment showed twice higher PAL levels than the control treatment in the lower stem. The responses at the first phase after NPF treatment in the upper stem and cotyledons were more moderate than in the lower stem.

Identification of scopolin and scopoletin

The changes of metabolite composition in the lower stem of morning glory cuttings that showed the largest changes in the PAL activity by NPF treatment were analyzed by reverse phase HPLC. Two compounds were found to increase rapidly after NPF treatment. The first was detected by UV monitoring the extract of the stems of cuttings that had been pretreated with NPF for 2 d. The accumulation level was about 3 times that in the stems of the water-pretreated cutting (data not shown). The isolated compound was demonstrated by NMR analysis to have a *cis*-olefin, an 1,2,4,5-tetrasubstituted benzene ring and a hexose moiety (Table I). The molecular weight was determined to be 354 g/mol by ESI-MS (m/z = 355 $[M+H]^+$). The hexose moiety was determined to be glucose by GC analysis of the acid-hydrolysate. The acid-hydrolysate also gave 7-hydroxy-6-methoxy coumarin, scopoletin, which was determined based on ^{1}H NMR spectra. From these data, the compound was identified as mono-β-glucopyranoside of scopoletin, scopolin (structures in Fig. 2). The second compound was detected by fluorescence monitoring. This compound was less polar than the first one and showed a more rapid increase in

Table I. Chemical shifts (ppm) of scopolin in pyridine-d_5.

^{13}C (75 MHz)		^{1}H (300 MHz)	
Sugar	Aglycon	Sugar	Aglycon
	161.1		
	151.2		
	150.1		
	147.0		
	143.7		7.67 d (J = 9.5)[a]
	114.1		6.34 d (J = 9.5)
	113.0		
	110.0		7.04 s
	104.2		7.51 s
101.8		5.79 d (J = 7.0)	
		5.03 br (hydroxy groups)	
79.1		4.19 ddd (J = 9.6, 5.0, 2.1)	
78.5		4.40 dd (J = 7.0, 6.3)	
74.7		4.42 dd (J = 8.5, 6.3)	
71.1		4.35 dd (J = 9.6, 8.5)	
62.3		4.55 dd (J = 12.0, 2.1)	
		4.41 dd (J = 12.0, 5.0)	
		56.2	3.74 s

[a] Coupling constant J (Hz).

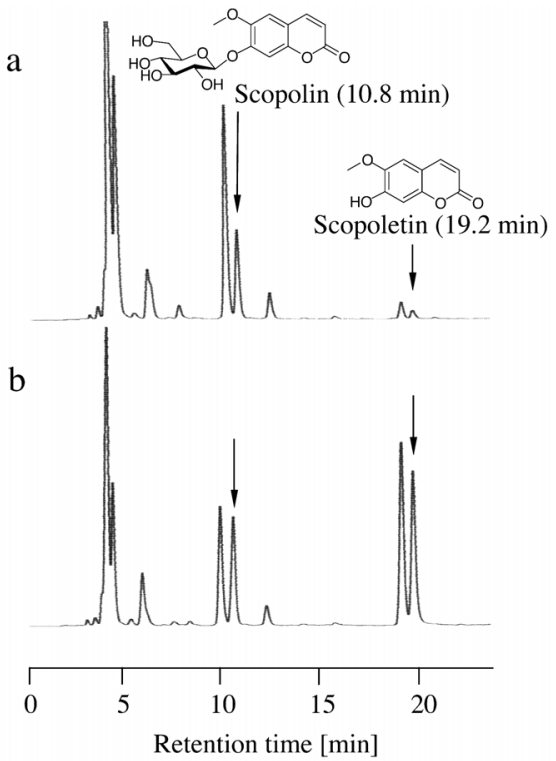

Fig. 2. Chromatograms of an extract from lower stems of the cuttings after treatment with distilled water (a) or NPF bud-cell suspension at 10^8 bud-cells/ml (b) for 12 h. Arrows indicate peaks of scopolin and scopoletin.

NPF-pretreated cuttings than that in the control plant after 12 h (Fig. 2). This constituent was identified to be scopoletin by co-chromatography and LC/ESI-MS with the standard sample.

Changes in the level of scopoletin and scopolin in the cutting of morning glory

The levels of scopoletin in the intact cuttings were 0.44, 0.43 and 0.11 nmol/g FW in the lower and upper stem and cotyledons, respectively. Scopolin was contained at 0.08, 2.21 and 0.59 nmol/g FW in the lower, upper stem and cotyledons of intact cuttings, respectively.

The levels of these compounds in morning glory rose in all parts of morning glory after NPF treatment. The greatest increases were observed in the lower stem.

The level of scopoletin in the lower stem began to increase at 3 h after NPF treatment, whereas that of the control cutting began to increase at 20 h (Fig. 3c). After a significant rate of increase around 6–12 h, the level of scopoletin in the lower stem of the NPF-treated cuttings reached 12-fold of that in the lower stem of the control cuttings at 20 h. The level of scopoletin in the lower stem of the cuttings treated with NPF kept on increasing between 20–48 h. The control cuttings showed a slight increase between 20–48 h. The similar profiles of increasing in the level of scopoletin were seen in the upper stem (Fig. 3b) and cotyledons (Fig. 3a).

The level of scopolin in the lower stem of the cuttings treated with NPF began to rise at 6 h and kept on rising between 6–48 h (Fig. 3f). A 6.7-fold increase in the scopolin level in the lower stem of the control cuttings was observed in cuttings treated with NPF at 20 h. A slight increase in the lower stem of the control cutting began after 12 h. The increase of the scopolin level in the lower stem of the control cuttings was more moderate than that of the cuttings treated with NPF. Similar changes in the level of scopolin were also seen systemically, in the upper stem and cotyledon (Fig. 3e and 3d, respectively).

Treatment with the supernatant of the NPF culture (10^8 bud-cells/ml), which did not contain NPF bud-cells, induced accumulation of scopoletin in the lower stem (Fig. 4). This inducing-activity of the supernatant was negated by autoclaving. A dead bud-cell suspension (10^8 bud-cells/ml) was also incapable to induce the accumulation of scopoletin in the lower stem.

The lower stem of cuttings treated with a PF bud-cell suspension at 10^5 bud-cells/ml for 20 h accumulated scopoletin and scopolin comparably with that of cuttings treated with a NPF suspension at 10^5 bud-cells/ml for 20 h (Table II). The scopolin accumulation in the lower stem of PF-treated cuttings at 10^8 bud-cells/ml for 20 h was smaller than that in the lower stem of the control cuttings. Furthermore, a severe withering was seen in cotyledons and the stem of the PF-treated cuttings at 10^8 bud-cells/ml.

Antifungal activity of scopoletin and scopolin

The germination rates of PF bud-cells in the presence of scopoletin (100, 10 μg/ml) or scopolin (100, 10 μg/ml) are shown in Table III. Both compounds had little inhibitory effect on the germination of PF bud-cells.

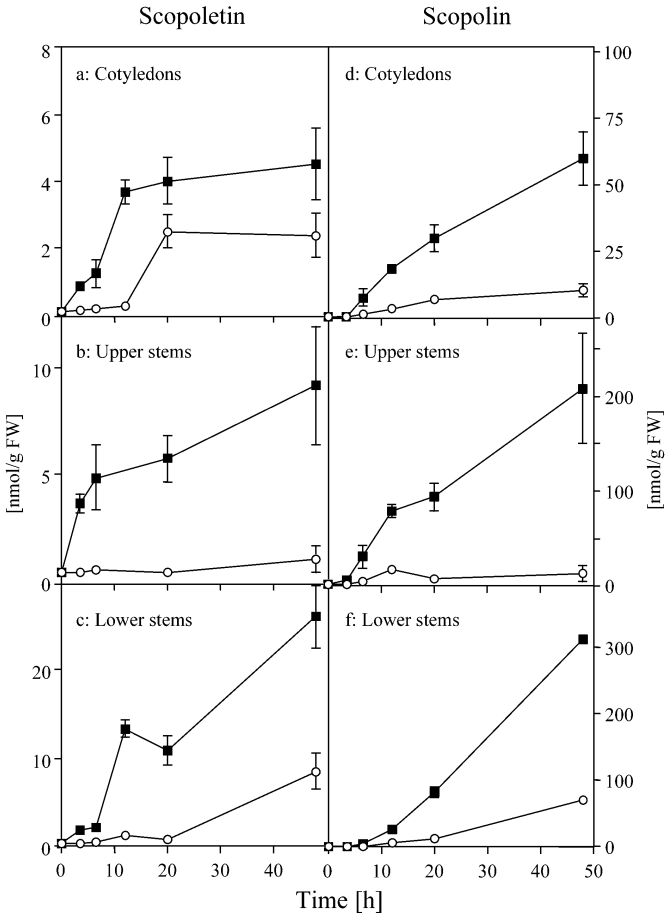

Fig. 3. Changes in scopoletin and scopolin content in the cuttings of morning glory, control (○) or NPF-treated (■). The cuttings were treated from 0 h and transferred to distilled water after 20 h. NPF was given at 10^8 bud-cells/ml. The data show the average of scopoletin content in tissues of the cuttings. The horizontal axis shows the period of treatment. The left column shows the scopoletin content in each portion: cotyledons (a), upper (b), and lower stems (c). The right column shows scopolin content in each portions: cotyledons (d), upper (e), and lower stems (f). Bars show standard errors (n = 3).

Effect of scopoletin and scopolin treatment on wilt symptom caused by PF

The preventive effect on etiolation of the cotyledons was evaluated using the chlorophyll content in cotyledons of cuttings as an index of disease severity. The chlorophyll content in cotyledons of cuttings that had been pretreated with water before PF treatment reduced to 0.83 mg/g FW after PF treatment for 7 d. This reduction of chlorophyll content in cotyledons of cuttings was cancelled by NPF pretreatment before PF treatment. The chlorophyll content in cotyledons of cuttings which had been pretreatment with NPF before PF treatment was 1.94 mg/g FW. No protective effect was observed by pretreatment with scopoletin or scopolin before PF treatment (Table IV). The chlorophyll content in cotyledons of cuttings that had been pretreated with scopoletin or scopolin solution at a range of concentrations (1–100 μg/ml) reduced in similar manner to that of the control cuttings.

Discussion

It has been shown that stimulation of phenylpropanoid metabolism gives rise to the accumulation of phenolics (Ni *et al.*, 1996) and cell wall lignification (Keller *et al.*, 1996; Ralph *et al.*, 1998) that operates against lytic enzymes of pathogens. The rapid induction of PAL suggests that changes in the phenylpropanoid metabolite profile occur in NPF-pretreated cuttings of morning glory.

Among phenylpropanoids, scopoletin and its monoglucoside, scopolin, were found to increase rapidly after NPF treatment. Induction of these compounds was seen not only in the lower stem but also in the upper stem and cotyledons of morning glory cuttings. Since mycelia of the

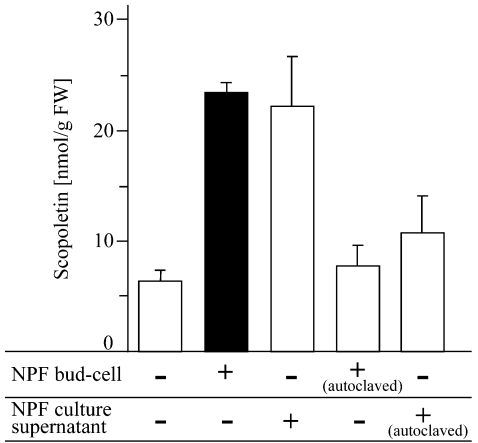

Fig. 4. Scopoletin content in lower stems of the cuttings treated with the supernatants of NPF culture and dead NPF bud-cells. Each treatment was given for 20 h. Values show the averages of scopoletin content in lower stems (bar: SE, n = 3). Viable and dead NPF bud-cells are given at 10^8 bud-cells/ml. The NPF culture containing 10^8 bud-cells/ml or its supernatant after centrifugation at 15,000 × *g* for 20 min was autoclaved.

Table II. Scopoletin and scopolin contents in the lower stem of treated morning glory cuttings.

Treatment	Bud-cells/ml	Scopoletin [nmol/g FW]	Scopolin [nmol/g FW]
NPF	10^8	21.2 ± 3.1	128.0 ± 18.1
	10^5	11.1 ± 3.3	62.0 ± 12.4
PF	10^8	nd[a]	14.4 ± 2.3
	10^5	6.6 ± 0.7	72.0 ± 3.7
Control		2.9 ± 0.5	48.0 ± 3.8

Each treatment was provided for 20 h with the nutrition solution. Control cuttings were treated with distilled water. The averages of scopoletin and scopolin content (nmol/g FW) in the lower stem of the treated cuttings are shown with the standard error (n = 3).

[a]nd: not determined.

Table III. Germination rates of PF in solutions of scopoletin and scopolin.

Treatment	Concentration [μg/ml]	Germination (%)
Scopoletin	10	74.3
	100	72.9
Scopolin	10	68.7
	100	88.9
Control		71.6

Each experiment contained 1% (v/v) ethanol and 1% (w/v) sucrose for 24 h in the dark at 25 °C. PF bud-cell concentration was at 10^5 bud-cells/ml.

Table IV. Chlorophyll content in cotyledons of cuttings after PF-treatment.

Pretreatment	Concentration [μg/ml]	Chlorophyll [mg/g FW]
Scopoletin	100	1.03 ± 0.18
	10	0.79 ± 0.16
	1	0.87 ± 0.05
Scopolin	100	0.78 ± 0.05
	10	0.91 ± 0.05
	1	0.73 ± 0.02
Control		0.83 ± 0.11
NPF[a]		1.94 ± 0.15

Pretreatments were provided for 20 h with the nutrition solution. Control cuttings were treated with distilled water. The average of chlorophyll content in cotyledons of cuttings treated with PF bud-cell suspension (10^5 bud-cells/ml) for 7 d is shown with the standard error (n = 3). Each treatment solution contained 1% (v/v) ethanol.

[a] NPF bud-cell suspension (10^8 bud-cells/ml).

treated NPF do not penetrate into cotyledon tissue (Shimizu *et al.*, 2000), the increase of scopoletin and scopolin level in this remote tissue might be due to the transfer from the lower part. Alternatively, a signal transducing mechanism could be present in morning glory, activating the biosynthesis of scopoletin and scopolin in remote tissue.

It has been reported that scopoletin exhibits antifungal activity against pathogens in some cases (Modafar *et al.*, 1993; Garcia *et al.*, 1995; Valle *et al.*, 1997). However no suppressive effect was observed on spore germination of PF in this study and it is unlikely that the antifungal activity of scopoletin plays a major role in the induced resistance by NPF. In tobacco plants, downregulation of salicylic acid – and pathogen-inducible UDP-glycosyltransferase, which acts efficiently on scopoletin, – causes reduction of scopoletin associated with weakened resistance to infection with TMV (Chong *et al.*, 2002). Although no protective effect of scopoletin on morning glory cutting was observed in this study, there might still be some room for verifying the relationship between salicylate and scopoletin.

The NPF culture supernatant induced accumulation of scopoletin to a level comparable to that of NPF bud-cell suspension, while the autoclaved supernatant of the NPF culture was incapable to induce scopoletin accumulation. Dead NPF bud-cells had no induction activity on accumulation of

scopoletin either. This is in agreement with the case of the induction of the resistance against PF in morning glory (Shimizu *et al.*, 2000). Therefore, it is very likely that the factor(s) responsible for the induction of scopoletin accumulation and the resistance against PF is the same.

Treatment with the PF bud-cell suspension at 10^5 bud-cells /ml caused an accumulation of scopolin after 20 h to a level comparable with that after the treatment with NPF bud-cell suspension at 10^5 bud-cells /ml. On the other hand, treatment with PF at 10^8 bud-cells/ml for 20 h caused severe withering of cotyledons and stem, in which the level of scopoletin is almost the same as that in the control cuttings. This indicates that PF has both the scopoletin (or resistance) inducing factor(s) and virulence factor(s) and the disease establishment by PF possibly depends on whether the virulence of PF can overcome the resistance induced by PF itself. Such virulence factor(s) may include toxin(s), lytic enzyme(s), as well as suppressor(s). Analysis of these virulence factor(s) of PF against morning glory is vital for elucidating the interactions between morning glory, NPF and PF.

Our study has shown that the rapid activation of the phenylpropanoids pathway is accompanied by induction of resistance in morning glory after treatment with NPF. The rapid accumulation of scopoletin and scopolin could be a facile marker of the interactions between morning glory and NPF. The ease of analysis of these compounds would facilitate the search for the factor(s) related to induction of resistance and to understand the chemical basis on which the induction mechanism of the resistance is established in this system.

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