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Chrysophanol is involved in the biofertilization and biocontrol activities of *Trichoderma*

Shu-Ying Liu [a], Chuan-Kai Liao [b], Chaur-Tsuen Lo [c], Hsueh-Hui Yang [d], Kuo-Chih Lin [b, **], Kou-Cheng Peng [b, *]

[a] Department of Molecular Biotechnology, Da-Yeh University, Changhua, Taiwan, ROC
[b] Department of Life Science, National Dong Hwa University, Hualien, Taiwan, ROC
[c] Department of Biotechnology, National Formosa University, Yunlin, Taiwan, ROC
[d] Department of Research, Buddhist Tzu Chi General Hospital, General Education Center, Tzu Chi College of Technology, Hualien, Taiwan, ROC

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ABSTRACT

The effect of chrysophanol, a secondary metabolite of *Trichoderma harzianum*, on the growth and disease resistance of cabbage against *Botrytis cinerea* was investigated. Chrysophanol assisted cabbage growth and promoted resistance against *B. cinerea*. Proteins related to photosynthesis were expressed in *T. harzianum*- and chrysophanol-treated cabbages. The transcription levels of photosynthesis- and sucrose transport-related genes increased in chrysophanol-treated cabbages. The transcription levels of chitinase increased in *T. harzianum*-treated cabbages and those of ascorbate peroxidase in *T. harzianum*- and chrysophanol-treated cabbages. Chrysophanol is involved in stimulating plant growth and photosynthesis and in eliciting host defense responses during the colonization of *T. harzianum*.

1. Introduction

Trichoderma spp. have been studied widely because of their ability to promote plant growth, stimulate plant disease resistance against pathogens, and provide broad-spectrum antagonistic activities against various soil-borne pathogens [1–3]. Therefore, *Trichoderma* spp. are marketed as biofertilizers and biopesticides [1,2]. In addition to the enzymes produced by *Trichoderma* spp. such as chitinases, glucanases, and proteases which are involved in the cell wall degradation of soil-borne pathogens, recent investigations have shown that the proteins such as small protein 1 (Sm1) and L-amino acid oxidase of *Trichoderma* provide high levels of protection to plants against phytopathogens by activating plant defense mechanisms [1,3–9].

Trichoderma spp. secrete a diverse range of secondary metabolites [10,11]. However, the *in vitro* production of secondary metabolites varies according to the species and strains of *Trichoderma*, state of the fungi, phytopathogens used for elicitation, media used for culturing, and balance between elicited biosynthesis and biotransformation rates of metabolites [11,12]. Certain secondary metabolites function as growth regulators and/or are involved in biocontrol. The secondary metabolites confer biocontrol activity either by directly inhibiting pathogen infection (direct antagonism) or by indirectly inducing host plant resistance [1].

Our laboratory previously isolated secondary metabolites such as chrysophanol and pachybasin from the cultures of *Trichoderma harzianum* strain ETS-323 and chrysophanol, pachybasin, emodin, ω-hydroxypachybasin, 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone from the cultures of strain Th-R16 [13,14]. Although chrysophanol, pachybasin, and emodin have been isolated from various species of *Trichoderma* spp. [10], the studies by Liu et al. [13,14] reported for the first time the isolation of these metabolites from the species of *T. harzianum*. Among these anthraquinones, chrysophanol and pachybasin are the predominant metabolites produced by these strains. The anthraquinones containing higher oxidation numbers have more effective antifungal activity, and their activities are concentration-dependent [14]. Moreover, pachybasin and emodin were found to be involved in the

* Corresponding author.
** Corresponding author.
E-mail addresses: kclin@mail.ndhu.edu.tw (K.-C. Lin), kcpeng@mail.ndhu.edu.tw (K.-C. Peng).

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self-regulation of *Trichoderma hazianum* mycoparasitic coiling [15]. In this study, we assessed the effect of chrysophanol on cabbage growth and performed a 3-way interaction comparing it with the *T. harzianum* strain ETS-323 to acquire a more appropriate understanding of the involvement of metabolites as growth regulators and/or in biocontrol mechanisms in *T. harzianum*. The effects of chrysophanol on stimulating disease resistance, protein production, and photosynthesis-, sucrose transport-, and defense-related gene expression in cabbage seedlings in response to *Botrytis cinerea* infection were determined and the functional roles of chrysophanol in *T. harzianum* were discussed.

2. Materials and methods

2.1. Chemicals

All chemicals including chrysophanol (product number 01542) were purchased from Sigma Aldrich (MO, USA), unless otherwise indicated. The chemical structure of the chrysophanol purchased from Sigma is identical to that of the chrysophanol produced by *T. harzianum* ETS-323 as determined by NMR [13].

2.2. Preparation of fungal material

T. harzianum ETS-323 was cultivated as described by Tseng et al. [16]. Briefly, *T. harzianum* ETS-323 was grown on potato dextrose agar (PDA, Difco Laboratories, MD, USA) plates at 27 °C for 5 d. The conidia on the plates were collected by flushing with 10 mL of sterile ddH_2O, and the conidia suspension was adjusted with sterile ddH_2O to a final concentration of 2.5×10^5 conidia/mL. Three milliliter of the *Trichoderma* spore suspension were used for inoculating cabbage seedlings. *B. cinerea* B134 was grown on PDA plates at 25 °C for 7 d.

2.3. Preparation of cabbage seedlings

The seeds of cabbage (*Brassica oleracea* var. *capitata*) were purchased from the Farmer Friend seed company (Pingtong, Taiwan, ROC). The seeds were pretreated with 0.1% chlorine for 10 min before receiving several washes of sterilized ddH_2O. The seeds were then placed on a filter paper moistened in water and kept in a petri dish at 24 °C in the dark for 36 h. Each germinated seed was grown in a round pot (4.5 × 3.5 cm) filled with potting soil (Floradur®, Floragard, Germany) and incubated in a 16-h light/8-h dark cycle at 24 °C for 30 d.

2.4. Three-way interactions

For the 3-way interaction studies, 30-day-old cabbage seedlings were separately predisposed to 3 mL of ddH_2O (regarding as a control) for 3 d, 3 mL of *Trichoderma* spore suspension (2.5×10^5 conidia/mL) for 3 d, or 3 mL of 20 μM chrysophanol (equivalent to a total of 15 μg) for 24 h by soil drenching in each pot. Five cabbage seedlings were used for each treatment, and all the treated cabbage seedlings were incubated in a humid chamber at 24 °C at 16-h light/8-h dark cycles. Afterward one of the second pair of true leaves was inoculated with a disc of agar containing *B. cinerea*, and the seedlings were simultaneously incubated in the humid chamber at 24 °C at 16-h light/8-h dark cycles for 3 d. The disease area was determined by using ImageJ software (http://imagej.net/Welcome). Relatively disease area was obtained by normalizing the disease area of control seedling. The uninfected leaf that is opposite to the infected leaf was collected for two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry and for reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analyses. The experiments were repeated for three times.

2.5. Protein preparation

We collected the uninfected leaf (approximately 100 mg) that was opposite to the *B. cinerea*-infected leaf in the three-way interaction. The samples were pulverized by applying liquid N_2 in a mortar and then adding 1 mL of ice cold methanol that contained 1 μL protease inhibitor cocktail solution (300 mg/mL). The samples were pelleted at 16000 × g for 5 min at 4 °C. The pellets were washed with 1 mL of ice cold methanol for 3 to 4 times and then washed with 1 mL of ice cold acetone. The pellets were then centrifuged at 16000× *g* for 5 min at 4 °C and dried by the Speedvac centrifugal dryer (Thermo, Victoria, Australia) for 3 min. The acetone-free pellet was rehydrated with rehydration buffer (9 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 1% dithiothreitol (DTT), 1 μL protease inhibitor cocktail solution. The rehydrated material was centrifuged at 16000× *g* for 30 min at 4 °C. The supernatant was collected and stored at −20 °C until use. The protein concentration was determined using the 2D-Quant Kit (Amersham/GE Healthcare, USA) according to the manufacturer's instructions.

2.6. The two-dimensional gel electrophoresis

The 2-DE was performed as previously described [16]. Isoelectric focusing was performed using 17-cm immobilized-pH-gradient (IPG) strips (Bio-Rad, CA, USA) with a pH range from 4 to 7. The protein samples were rehydrated in a solution that contained 8 M urea, 2% CHAPS, 50 mM DTT, and 2% carrier ampholyte (Bio-Rad, CA, USA). Approximately 100 mg of the protein samples were loaded in the focusing tray and allowed to be absorbed onto the gel strip (12 h actively with a 50 V current). IEF was performed in the PROTEAN IEF Cell System (Bio-Rad, CA, USA). IPG strips were focused up to a total of 14 kV h by using a three-step program (i.e., 250 V for 1 h, 4 kV for 3 h, and progressive increases in voltage until 10 kV h was reached). The strips were equilibrated in a solution of 6 M urea, 0.05 M Tris-HCl (pH 8.8), 1% sodium dodecyl sulfate (SDS), 30% glycerol, and 1% DTT for 15 min followed by 15 min in 6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 30% glycerol, and 4% iodoacetamide. For the second separation, the strips were positioned on 1-mm thick SDS polyacrylamide gels (10%) and sealed with 1% agarose. The gels were run on the PROTEAN II system (Bio-Rad, CA, USA) at 25 mA gel^{-1} until the bromophenol blue dye front migrated to the end of the SDS gel. For protein visualization and image analysis, a silver staining of the gel that was compatible with mass spectrometry was performed as described by Gharahdaghi et al. [17] with slight modification. The gel was fixed for 30 min in 50% methanol and 10% acetic acid. The gels were then stained with the Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad, CA, USA) following the manufacturer's instruction.

2.7. In-gel digestion and electrospray ionization (ESI)-QUAD-time of flight (TOF) mass spectrometry

Protein in-gel digestion and ESI-QUAD-TOF mass spectrometry were performed as previously described [16].

2.8. Total RNA extraction

Total RNA was extracted from approximately 100 mg of leaf tissue using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Briefly, approximately 100 mg of leaves were homogenized with 1 mL of TRIzol reagent. The RNA