JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Involvement of Pachybasin and Emodin in Self-Regulation of *Trichoderma harzianum* Mycoparasitic Coiling

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Supporting Information

ABSTRACT: Our aim was to determine the effects of two secondary metabolites secreted by *Trichoderma harzianum*, pachybasin and emodin, on the mycoparasitic coiling behavior and cAMP content of *T. harzianum*. The number of *T. harzianum* coils around Nylon 66 fiber was increased in the presence of *R. solani*. The number of *T. harzianum* coils around *R. solani* hyphae and Nylon 66 fiber were significantly increased in the presence of pachybasin and emodin. The cAMP level in *T. harzianum* was significantly increased by close contact with *R. solani* and much higer cAMP level in the presence of exogenous pachybasin and emodin. A cAMP inhibitor diminished the effect of pachybasin and emodin on *T. harzianum* coiling around Nylon 66 fiber. The results suggest that pachybasin and emodin mediate the increase in the number of *Trichoderma* mycoparasitic coils via cAMP signaling. This is the first report to suggest that pachybasin and emodin play roles in the biocontrol mechanism of *Trichoderma*.

KEYWORDS: *cAMP, coiling, emodin, pachybasin, self-regulation, Trichoderma*

INTRODUCTION

Trichoderma spp. are important biological control agents used in agricultural settings against phytopathogenic fungi such as *Rhizoctonia solani* and *Botrytis cinerea*. *Trichoderma* mycoparasitism is a complex process that involves recognition, attack, and subsequent penetration and killing. The attack involves degradation of the fungal cell wall followed by the dissimilation of its cellular content,[1,2] production of antibiotic compounds,[3−5] changes such as regulation of cAMP signaling to induce coiling around the phytopathogenic fungal hyphae, and development of appressorium-like structures.[6,7]

Fungi produce a wide range of secondary metabolites which may not have any apparent biological functions or are not essential to normal metabolism. Some secondary metabolites act as communication signals between the producer organism and other living beings, such as microorganisms, plants, or animals. Examples of these secondary metabolites include antibiotics, mycotoxins, plant growth factors, fungal elicitors, and host plant and animal defensins.[8,9] Among these, pachybasin (1-hydroxy-3-methylanthraquinone, Figure 1A) and emodin (6-methyl-1,3,8-trihydroxyanthraquinone, Figure 1B) represent important secondary metabolites of *Trichoderma*.[5,10,11] These compounds are active against phytopathogenic fungi, such as *R. solani* and *B. cinerea*.[5]

Self-regulation is a state that a mycelium adjusts by altering its internal partitioning, penetration, and deformation to facilitate its adaption to the circumstances.[12] Many fungal secondary metabolites act as self-regulating factors such as butyrolactone-containing molecules, melanin, linoleic-acid derived psi factor, and zearalenone.[8] Butyrolactone I (α-oxo-β-(*p*-hydroxyphenyl)-γ-(*p*-hydroxy-*m*-3,3-dimethylallyl-benzyl)-γ-methoxycarbonyl-γ-butyrolactone, Figure 1C) produced by

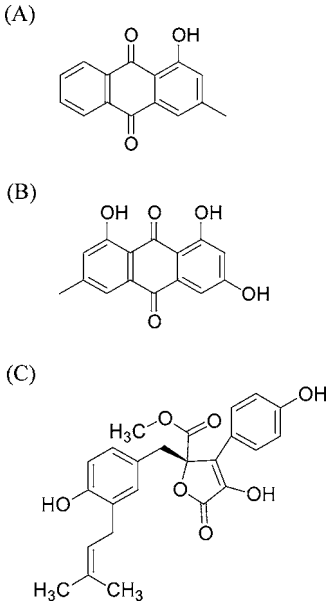

Figure 1. Structures of (A) pachybasin, (B) emodin, and (C) butyrolactone I.

organisms, such as *Aspergillus* spp. and *Streptomyces* spp., cause the morphogenesis of aerial mycelium into spores. In *Colletotrichum lagenarium* and *Venturia inaequalis*, melanin

Received: July 12, 2011
Revised: January 5, 2012
Accepted: January 31, 2012
Published: January 31, 2012

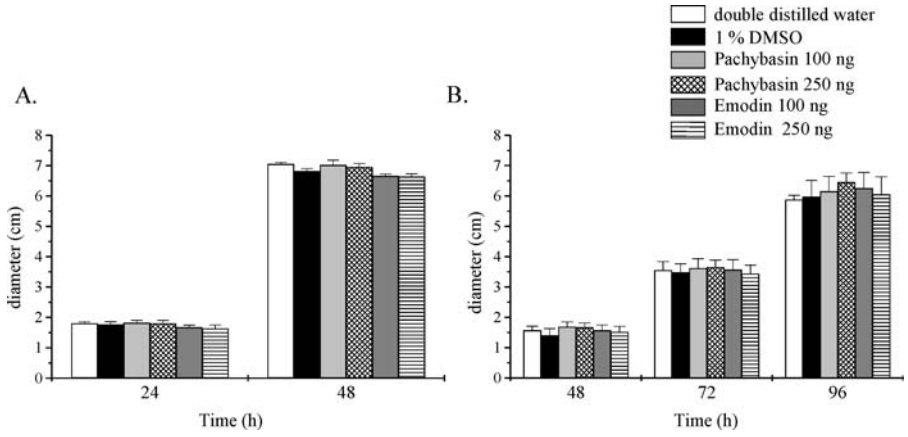

Figure 2. Effects of pachybasin and emodin on the mycelial growth and colony morphology of *T. harzianum* and *R. solani*. (A) The colony diameter of *T. harzianum* was measured at 24 and 48 h. (B) The colony diameter of *R. solani* was measured at 24, 48, and 96 h. Values are means ± SEM (n = 6).

biosynthesis has been associated with the formation of appressoria.[13−15] Linoleic-acid-derived psi factor and zearalenone induce sporulation in *Aspergillus nidulans* and *Fusarium graminearum*, respectively.[16−18]

In *Trichoderma*, the cAMP signaling pathway is involved in secretion of hydrolytic enzymes, antibiotic production, and coiling around host hyphae.[1] Application of exogenous cAMP or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) is reported to increase the number of coils wrapped around biomimetic fibers.[19] In subgroup I Gα subunit Tga3 mutants (Δ*tga3*) with reduced intracellular cAMP concentration, no mycoparasitic structures are formed.[20]

The mechanism of biocontrol by two major secondary metabolites, pachybasin and emodin, secreted from *Trichoderma* is of interest. In the present study, the effects of exogenous pachybasin and emodin on coiling behavior and cAMP level in *T. harzianum* were examined. The results showed that pachybasin and emodin facilitated the adaptability of *T. harzianum* to its environment by increasing mycoparasitic coil density and cAMP level.

MATERIALS AND METHODS

Materials. Pachybasin was isolated from *T. harzianum* ETS323, as previously described.[10] We obtained emodin, atropine, diethyl ether, and dimethyl sulfoxide (DMSO) from Sigma Chemical Company (St. Louis, MO), lactophenol blue solution from Fluka (Buchs, Switzerland), potato dextrose agar (PDA) from Becton Dickinson (Franklin Lakes, NJ), cover glasses from Marienfele (Lauda-Königshofen, Germany), Nylon 66 fiber from the Acelon Chemicals & Fiber Corporation (Chang Hua, Taiwan), cAMP enzyme immunoassay (EIA) kit from the Cayman Chemical Company (Ann Arbor, MI), and Coomassie Plus protein assay reagent from Thermo Scientific (Rockford, IL).

Mycelial Growth and Colony Morphology. The effects of each tested compound on growth rate were determined by placing a 1.5 mm diameter mycelial plug of *T. harzianum* or *R. solani* in the center of a PDA plate. A 10 μL sample of a solution of each compound (10 or 25 ng/μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. The plugs were maintained at 28 °C in the dark, and the growth diameter was recorded every 24 h.

Mycoparasitic Coiling. A 1.5 mm diameter plug of *T. harzianum* and a 1.5 mm diameter plug of *R. solani* were placed at opposite ends of a 24 × 32 mm^2 cover glass coated with 40% PDA (PDA-covered cover glass). A 10 μL sample of a solution of each compound (10 or 25 ng/μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. Then, the cover glass was placed in a sterile Petri dish containing sterilized and moistened cotton and incubated at 28 °C in the dark. Time zero was defined as the time when the hyphae of the two fungi first make contact. Lactophenol cotton blue (50%) was added to stain the cell wall and make the number of *T. harzianum* coils around *R. solani* easier to count under the light microscope (Olympus IX70, Tokyo, Japan). Sterile Nylon 66 fibers were used as a biometric system as previously described with slight modification.[21] A 1.5 mm diameter plug of *T. harzianum* was placed on one end of PDA-coated cover glass. A 10 μL sample of a solution of each compound (10 or 25 ng/μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. After 24 h, the Nylon 66 fibers were placed in front of the advancing *T. harzianum* mycelia. Another 24 h later, the number of *T. harzianum* coils on the Nylon 66 fibers was counted under the light microscope.

On the other hand, a 1.5 mm diameter plug of *T. harzianum* and a 1.5 mm diameter plug of *R. solani* were placed at opposite ends of a 24 × 32 mm^2 PDA-coated cover glass. A 10 μL sample of a solution of each compound (10 or 25 ng/μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. After 24 h of incubation, Nylon 66 fiber was placed between the two plugs. Another 24 h later, the distance between *T. harzianum* and *R. solani* should be greater than 5 mm. At that time, the number of *T. harzianum* coils around Nylon 66 fibers was counted in 3−4 randomly chosen fields within the interactive zone under the light microscope. The effect of the cAMP inhibitor, atropine, on *T. harzianum* coiling was determined in PDA medium containing 1 mM atropine. A 10 μL sample of a solution of each compound (10 or 25 ng/ μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. After 24 h of incubation, the Nylon 66 fibers were placed in front of the advancing *T. harzianum* mycelia, and after another 24 h of incubation, the number of *T. harzianum* coils were counted as before.

cAMP Concentration Assay. Several 1.5 mm diameter plugs of *T. harzianum* alone were placed on one end of a cellophane membrane overlaying a PDA plate. A 10 μL sample of a solution of each compound (10 or 25 ng/ μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. After ∼60 h of incubation, the mycelia were harvested and stored at −80 °C until the assay. The mycelia were mixed with 400 μL of 5% trichloroacetic acid (TCA), homogenized by sonication, and then centrifuged (1500g for 10 min at 4 °C). The supernatant was collected and extracted 3 times with water-saturated diethyl ether to eliminate the TCA. The diethyl ether was evaporated by heating the sample to 70 °C for 5 min. The cAMP level in each sample was determined using a cAMP EIA kit according to manufacturer's instructions. The cAMP concentrations were calculated from OD_{415} values recorded by a microplate reader (BIO-RAD Model 680,

Hercules, CA). In addition, *T. harzianum* and *R. solani* plugs were placed on opposite ends of cellophane covering PDA plates. A 10 μL sample of a solution of each compound (10 or 25 ng/μL) in 1% DMSO (1:100 DMSO/water v/v) or 10 μL of 1% DMSO (control solution) was applied to the surface of each plug. After ∼45 h of incubation, *T. harzianum* and *R. solani* were not in direct contact, and the distance between *T. harzianum* and *R. solani* was greater than 5 mm. After ∼60 h of incubation, *T. harzianum* and *R. solani* were in direct contact, and the zone of interaction was 5 mm wide. The cAMP level of *T. harzianum* after treatment with one of the test agents was determined using the above-described method. Protein concentration was determined using Coomassie Plus protein assay reagent.

Data Analysis. All data are expressed as means ± SEM. Statistical significance of the difference between groups was determined by one-way analysis of variance (ANOVA) or two-way ANOVA followed by a Student−Newman−Keuls posthoc test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Effects of Pachybasin and Emodin on Mycelial Growth and Colony Morphology of *T. harzianum* and *R. solani*. Under the culture conditions, *T. harzianum* and *R. solani* took more than 60 and 100 h, respectively, to fully cover a 90-mm Petri dish. The mycelial growth (Figure 2A,B) and colony morphology (Supporting Information (SI) Figure S1A,B) of both *T. harzianum* (Figure 2A and SI Figure S1A) and *R. solani* (Figure 2B and SI Figure S1B) were unaffected by treatment with pachybasin or emodin at all concentrations and by treatment with 1% DMSO.

Effect of Pachybasin and Emodin on *T. harzianum* Coiling around Nylon 66 Fibers. To examine the effects on coiling, the number of coils was counted after incubating *T. harzianum* with various concentrations of pachybasin and emodin. All concentrations caused the number of coils to increase (Figure 3 and SI Figure S2). The coil density was slightly higher under experimental conditions [*T. harzianum* before mycelial contact with *R. solani* (TR precontact)] than under control (1% DMSO) conditions [7.2 ± 0.2 coils mm^{-1} (SI Figure S2D) vs 4.3 ± 0.2 coils mm^{-1} (SI Figure S2A)]. Exogenous pachybasin (100 and 250 ng) increased the coil density to 9.5 ± 0.5 and 10.6 ± 0.6 coils mm^{-1}, respectively (Figure 3 and SI Figure S2B), while exogenous emodin (100 and 250 ng) increased the coil density to 8.2 ± 0.2 and 8.3 ± 0.4 coils mm^{-1}, respectively (Figure 3 and SI Figure S2C).

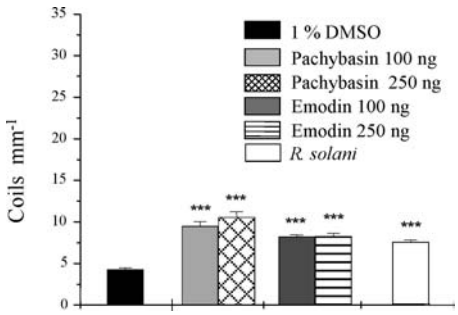

Figure 3. Effects of exogenous application of pachybasin and emodin to *T. harzianum* before or during hyphal contact with *R. solani* on the density of *T. harzianum* coils around Nylon 66 fibers. Evaluation was carried out by counting the number of coils produced by *T. harzianum* alone treated with 1% DMSO, pachybasin, or emodin or by *T. harzianum* before hyphal interaction with *R. solani*. Values are means ± SEM (n = 4). *** indicates P < 0.001 compared with the control (1% DMSO). Scale bar = 250 μm.

Effect of Pachybasin and Emodin Treatment on Attachment and Coiling of *T. harzianum* to *R. solani*. Apparent contact between hyphae of both fungi in coculture occurred within 40−45 h after inoculation (TR direct-contact). Although mixed, the hyphae of *T. harzianum* (thin) and *R. solani* (thick) can be easily distinguished morphologically. Initially, the hyphae of *T. harzianum* established contact with *R. solani* (the host) by coiling around its hyphae. The number of coils was counted in the interaction region at 2, 5, 8, and 12 h after contact was established. Under control conditions (1% DMSO), the number of coils mm^{-1} increased in a time-dependent manner [2−3, 6−8, 13−18, and 15−19 after contact for 2, 5, 8, and 12 h, respectively (Figures 4 and 5)]. After 12 h, the high density of coils and the distortion and wrinkling of the hyphae made counting impossible. The coil density at 12 h was increased from 3.42 ± 1.0 to 27.7 ± 2.2 coils mm^{-1} by pachybasin [100 and 250 ng (Figure 4A and SI Figure S3B,E)] from 6.7 ± 0.9 to 28.6 ± 2.4 coils mm^{-1} by emodin [100 and 250 ng (Figure 4B and SI Figure S3C,F)].

Effect of Pachybasin- and Emodin-Treated *R. solani* on *T. harzianum* Coiling. Specific recognition events as well as coil density were investigated with pachybasin- and emodin-treated *R. solani*. The application of pachybasin (100 and 250 ng) and emodin (100 and 250 ng) time-dependently increased coil density (in coils mm^{-1}: 2−3, 9−12, 14−18, and 15−21 after hyphal contact for 2, 5, 8, and 12 h, respectively). Coil densities were roughly the same under control conditions [with application of 1% DMSO (Figure 5 and SI Figure S4)].

Concentration of cAMP in *T. harzianum*. The concentration of cAMP was similar in *T. harzianum* alone (T, 128.2 ± 0.3 pmol mg^{-1}) and *T. harzianum* before contact with *R. solani* (TR precontact, 100.2 ± 1.1 pmol mg^{-1}) but increased significantly in *T. harzianum* in contact with *R. solani* (TR direct-contact, 318.4 ± 27.4 pmole mg^{-1}), in *T. harzianum* in contact with *R. solani* after addition of pachybasin (413.2 ± 33.0 pmol mg^{-1}), and in *T. harzianum* in contact with *R. solani* after addition of emodin (465.1 ± 8.8 pmol mg^{-1}). However, the addition of pachybasin (188.3 ± 38.3 pmol mg^{-1}) and emodin (185.5 ± 43.7 pmol mg^{-1}) to *T. harzianum* alone had no significant effect on cAMP concentration (128.2 ± 0.3 pmol mg^{-1}). The application of pachybasin (141.4 ± 21.9 pmol mg^{-1}) and emodin (157.0 ± 4.6 pmol mg^{-1}) to *T. harzianum* before contact with *R. solani* also had no effect on cAMP concentration [100.2 ± 1.1 pmol mg^{-1} (Figure 6)].

cAMP Inhibitor Reduced Pachybasin- and Emodin-Induced Increase in *T. harzianum* Mycoparasitic Coil Density around Nylon 66 Fibers. The addition of 1 mM atropine (a cAMP inhibitor) reversed pachybasin (250 ng)- or emodin (250 ng)-induced increase in coil density around Nylon 66 fibers [4.2 ± 0.3 coils mm^{-1} and 3.6 ± 0.4 coils mm^{-1} compared to 10.8 ± 0.1 coils mm^{-1} and 8.5 ± 0.1 coils mm^{-1}, respectively, for pachybasin alone and emodin alone (Figure 7)].

DISCUSSION

Anthraquinones are secondary metabolites of *Trichoderma* species.[5,10,22] While anthraquinones typically function as pigments, they have been reported to function as laxatives, diuretics, phytoestrogens, immune stimulators, antifungal agents, antiviral agents, and anticancer agents.[5,23−26]

Previous studies indicate that anthraquinones act against *R. solani* and *B. cinerea*,[5] but not at low concentrations. Mycoparasitic coiling is an important microscopic feature of

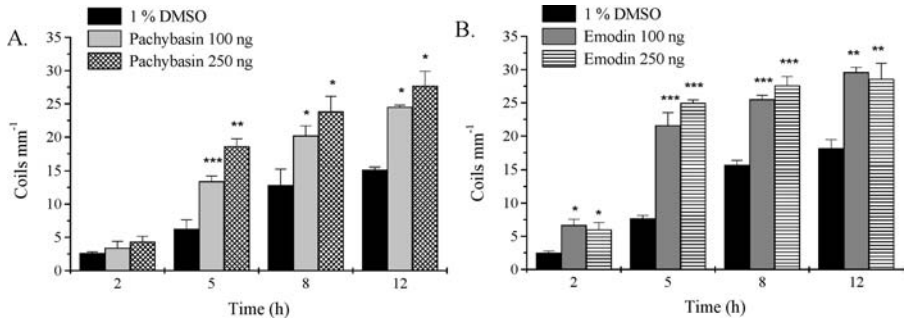

Figure 4. Effects of exogenous application of pachybasin and emodin to *T. harzianum* on the mycoparasitic coiling of *T. harzianum* around *R. solani*. Evaluation was carried out by counting the number of coils produced by *T. harzianum* after treatment of *T. harzianum* with pachybasin (A) or emodin (B). Values are means ± SEM (n = 4). * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ compared with control (1% DMSO). Scale bar = 250 μm.

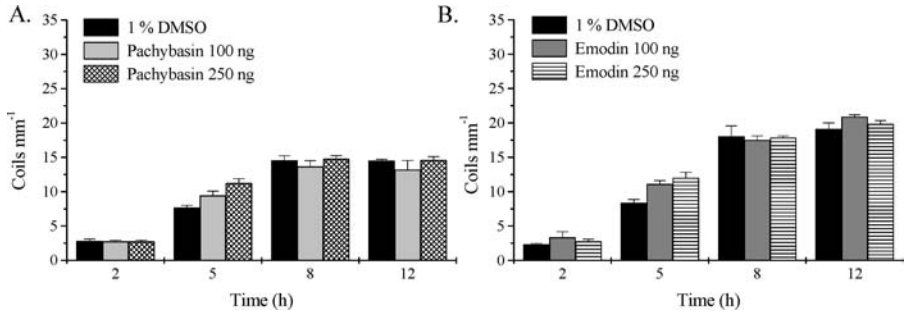

Figure 5. Effect of exogenous application of pachybasin and emodin to *R. solani* on mycoparasitic coiling of *T. harzianum* around hyphae of *R. solani*. Evaluation was carried out by counting the number of coils produced by *T. harzianum* after treatment of *R. solani* with pachybasin (A) or emodin (B). Values are means ± SEM (n = 4). Scale bar = 250 μm.

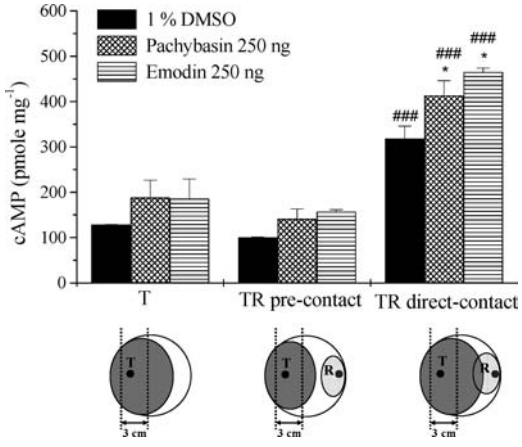

Figure 6. Effects of pachybasin and emodin on cAMP levels in *T. harzianum* alone or in *T. harzianum* after interaction with *R. solani*. Expression cAMP in *T. harzianum* treated with pachybasin and emodin at different stages. T: *T. harzianum* grown alone; *T. harzianum* grown with *R. solani* but at an early stage before direct contact between the two fungi (TR-precontact); *T. harzianum* grown with *R. solani* but at a later stage of direct contact with an interaction zone of 5 mm (TR-direct contact). Values are means ± SEM (n = 4). * indicates $P < 0.05$, compared with control (1% DMSO). ### indicates $P < 0.001$, compared with *T. harzianum* alone.

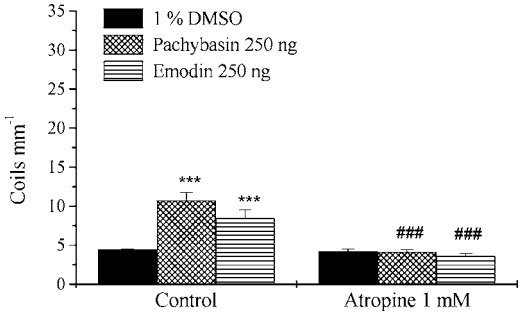

Figure 7. Effects of atropine on pachybasin- and emodin-induced increase in *T. harzianum* coil density around Nylon 66 fiber. *T. harzianum* was placed on PDA plates containing atropine. Evaluation was carried out by counting the number of *T. harzianum* coils after each treatment with pachybasin or emodin. Values are means ± SEM (n = 4). *** indicates $P < 0.001$, compared with the control (1% DMSO). ### indicates $P < 0.001$, compared with the control (1% DMSO).

the antagonism between *T. harzianum* and *R. solani*.[1] We reported here that low concentrations of pachybasin and emodin indeed increase the number of *T. harzianum* coils around the phytopathogen, *R. solani*, without affecting the growth rate and morphology of each species. Also, it was reported that the cAMP dependent pathway[7,19] and cAMP independent pathway[27] are involved in mycoparasitic coiling. In the former pathway, both G-protein activators, i.e., mastoparan and fluoroaluminate, and induction of the G-α gene (*tga1*) showed a positive relationship to higher intracellular cAMP concentration and number of mycoparasitic coils.[1] A G protein mutant of *T. atroviride* (Δ*tga3*) had reduced intracellular cAMP concentration and no ability to form coils but gained this ability after addition of exogenous cAMP.[20] In addition, the number of coils was increased by application of exogenous cAMP or the phosphodiesterase inhibitor, IBMX.[19] For the cAMP independent pathway, the number of coils were increased and concentration of cAMP decreased in *T. harzianum* over-

expressing the subgroup I G_α subunit.[27] Since pachybasin and emodin are the major secondary metabolites secreted by *T. harzianum*, it was of interest to determine whether pachybasin and emodin might self-regulate the coiling behavior of *T. harzianum* and determine the involvement of the cAMP pathway in this mechanism. First, the density of *T. harzianum* coils around Nylon 66 fiber increased under TR precontact conditions, compared with control (DMSO) conditions (Figure 3 and SI Figure S2D). Furthermore, there was no significant difference in cAMP concentration between *T. harzianum* under precontact conditions and *T. harzianum* under control conditions (Figure 6). These results suggested that the coiling behavior of *Trichoderma* is cAMP independent before *T. harzianum* interacts with *R. solani*. However, after *T. harzianum* interacts with *R. solani*, the density of coils around *R. solani* increased in a time dependent way (Figure 4, black columns) and the cAMP concentration also increased (Figure 6). These results suggested that cAMP might have a role in *T. harzianum* coiling around *R. solani*. These data strongly support the participation of both the cAMP-dependent and the cAMP-independent pathways in *Trichoderma* mycoparasitic coiling behavior. Also, it was also of interest to determine whether pachybasin or emodin could affect *T. harzianum* coiling and cAMP concentration in the absence of contact with *R. solani*. Pachybasin or emodin increased the density of *T. harzianum* coils around Nylon 66 fibers (Figure 3 and SI Figure S2B,C), but this effect was abolished by atropine, a blocker of cAMP production in fungi[28,29] (Figure 7). These results suggested that pachybasin and emodin behaved as self-regulating factors that activated the cAMP signaling pathway and upregulated mycoparasitic coiling of *T. harzianum* around Nylon 66 fiber, in the absence of *R. solani*. When *T. harzianum* and *R. solani* were in direct contact in the absence of Nylon 66 fiber, pachybasin and emodin treatments increased the coil density [~30 coils mm^{-1} in 12 h vs ~20 coils mm^{-1} under control (DMSO) conditions, in time dependent way, Figure 4] and cAMP concentration [compared with control (DMSO), Figure 6]. Therefore, the pachybasin- and emodin-induced increase in mycoparasitic coiling when *T. harzianum* and *R. solani* were in direct contact is a direct effect of cAMP. However, the pachybasin- or emodin-induced increase in coil density (Figure 4) was much greater under TR-direct contact conditions than under *T. harzianum*-Nylon 66 fiber direct contact conditions (Figure 3) for equivalent periods. So, both the presence of *R. solani* and of exogenous pachybasin and emodin were responsible for the increase in coil number. Moreover, the non-dose-dependency of the pachybasin- or emodin-induced increase in *T. harzianum* coil number in the presence of Nylon 66 fiber may be due the lack of*R. solani*. Taken together, these results indicate that *T. harzianum* produces pachybasin and emodin in order to enhance its ability to mycoparasitize *R. solani* through enhanced coil formation.

Trichoderma secretes secondary metabolites to facilitate its adaption to the environment. Therefore, pachybasin and emodin may regulate recognizable markers on *R. solani*. However, application of pachybasin and emodin to *R. solani* had no effect on mycoparasitic coil density under our assay conditions, suggesting that pachybasin and emodin may not be involved in *R. solani*-mediated recognition.

This study provides a new look at the biocontrol mechanism of *T. harzianum* mycoparasitic coiling. The upregulation of *Trichoderma* coil density by self-regulating factors, anthraquinones, was mediated through the cAMP signaling pathway. We also discovered that the transmission of signals mediating mycoparasitic coiling between *T. harzianum* and phytopathogenic fungi proceeds via both the cAMP-dependent and cAMP-independent pathways.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was supported by Grant NSC 99-2324-347B-259-001 and NSC 99-2313-B-212-001-MY3 from the National Science Council, Taiwan.

■ ACKNOWLEDGMENTS

We thank Dr. R. Y. Chang for generously providing equipment support.

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