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# Purification and Phytotoxicity of Apicidins Produced by the *Fusarium semitectum* KCTC16676

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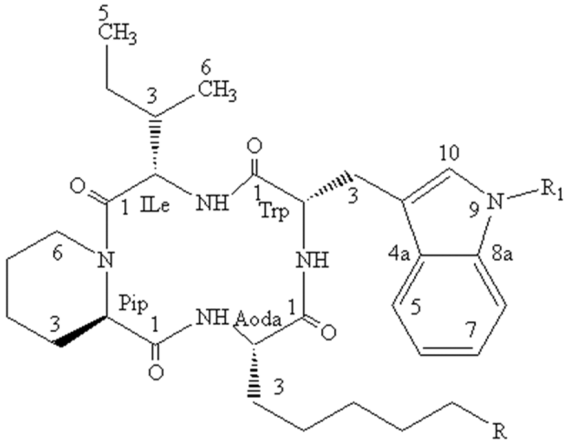
**Apicidin is a cyclic tetrapeptide produced by some *Fusarium* species and is known to inhibit Apicomplexan histone deacetylase. The goals of this study were to determine species identity of *Fusarium* isolate KCTC16676, an apicidin producer, to improve a method for apicidin extraction, and to test phytotoxicity of apicidin and its analogs. We compared sequences of the translation elongation factor 1-alpha (TEF) gene in KCTC16676 with those from isolates representing diverse *Fusarium* species, which showed that KCTC16676 belongs to the *F. semitectum-F. equiseti* species complex. To enhance apicidin production, after culturing isolate KCTC16676 on a wheat medium for 3 weeks at 25°C, the culture was extracted with chloroform. Apicidins were purified through a reverse phase $C_{18}$ silica gel column, resulting in 5 g of apicidin, 200 mg of apicidin A, and 300 mg of apicidin $D_2$ from 4 kg of wheat cultures; this represents a significant yield improvement from a previous method, offers more materials to study the modes of its action, and facilitates the elucidation of the apicidin biosynthesis pathway. Apicidin and apicidin $D_2$ showed phytotoxicity on both seedlings and 2-week-old plants of diverse species, and weeds were more sensitive to apicidins than vegetables**

***Keywords*** **:** apicidin, *Fusarium semitectum*, hemorrhagic factor, mycotoxin

The genus *Fusarium* is widely distributed in soil, aerial plant parts, plant debris, and other organic substrates, and can cause diseases in plants, humans, and domesticated animals (Leslie and Summerell, 2006). In addition, *Fusarium* spp. produce many secondary metabolites that are associated with plant disease, cancer and other growth defects in humans and animals (Desjardines, 2006). Some of the *Fusarium* secondary metabolites have been used commercially as plant and animal growth promoters (Leslie and Summerell, 2006).

*Fusarium* taxonomy has been plagued by rapidly changing species concepts, ranging from 9 to 1,000 species depending on the species concepts employed (Summerell et al., 2003). Although taxonomy based on the morphological species concept (Gerlach and Nirenberg, 1982; Nelson et al., 1983) was widely accepted, it failed to differentiate certain species due to their morphological complexity. Biological (Leslie, 2001) and phylogenetic (Nirenberg and O'Donnell, 1998) species concepts have also been applied, which in some situations better resolve taxonomic questions.

Apicidin is a cyclic tetrapeptide consisting of cyclo-{L-*N*-methoxyltryptophan-L-isoleucyl-D-pipecolinyl-L-(2-amino-8-oxodecanoyl)} (Fig. 1; Singh et al., 1996). Apicidin contains classical electrophilic keto group, and the




1. R=$COCH_2CH_3$ $R_1$=$OCH_3$
2. R=$COCH_2CH_3$ $R_1$=H
3. R=$CH(S\text{-}OH)CH_2CH_3$ $R_1$=$OCH_3$

**Fig. 1.** Structure of apicidin (1), apicidin A (2), and apicidin $D_2$ (3).

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cyclic nature of the peptide is critical for its biological activity (Singh et al., 2002). Apicidin exhibits potent, broad spectrum antiprotozoal activity *in vivo* against Apicomplexan parasites. Antiprotozoal activity of apicidin results from the inhibition of histone deacetylase, a key nuclear enzyme involved in transcriptional control (Darkin-Rattray et al., 1996). Apicidin also has showed anti-growth activity in HeLa cells, human endometrial cells and ovarian cancer cells (Hong et al., 2003; Ueda et al., 2007).

Several fungal cyclic tetrapeptides such as HC-toxin (Kawai et al., 1983; Liesch et al., 1982), Cly-1 (Takayama et al., 1984), and Cly-2 (Hirota et al., 1973) have been shown to be phytotoxic. These cyclic peptides, which were isolated from various fungal species, exhibit structures similar to that of apicidin. Phytotoxicity of apicidin was assayed on duckweed (*Lemna pausicostata* L.) in which apicidin resulted in disruption of plant cell membrane as well as the inhibition of histone deacetylase (Abbas et al., 2001). In addition, many organelles in apicidin-treated tissues appear less distinct, and chloroplasts contain larger starch grains than that of the control (Abbas et al., 2001).

We previously reported apicidin production by *Fusarium* sp. KCTC16676 and toxicity of apicidin in rat, brine shrimp and human tumor cells (Park et al., 1999). In that study, the species identity of strain KCTC16676 was not resolved due to its morphological ambiguity and phytotoxicity of apicidin on various plants was not tested due to the small amount of apicidin produced. Here, we characterized the species identity of KCTC16676 strain using a gene sequence, enhanced the extraction procedure to increase apicidin production, and tested phytotoxicity of apicidins on several plant species.

## Materials and Methods

**Strain and culture conditions.** *Fusarium* strain KCTC16676 was originally isolated from soybean seeds (Park et al., 1999). The strain was stored in 25% glycerol at −70°C and revitalized on potato dextrose agar (PDA) as needed. For genomic DNA extraction, it was grown in 50 ml of liquid complete medium (CM; Leslie and Summerell, 2006) in 250-ml Erlenmeyer flasks at 25°C for 3 days on a rotary shaking incubator at 150 rpm, and mycelia were harvested and lyophilized. For apicidin production, Erlenmeyer flasks (1 L) containing 200 g of wheat and 120 ml of deionized water were autoclaved at 121°C for 1 h. Agar blocks of KCTC16676 grown on PDA for 3 days were inoculated on the wheat medium, and the cultures were incubated for 3 weeks at 25°C. The cultures were air-dried for 5 days in a ventilated hood and were ground to the consistency of flour.

**Polymerase chain reaction (PCR) and sequencing.** A standard PCR protocol was used to amplify the translation elongation factor 1-alpha (TEF) gene region. TEF-F (5'-ATGGGTAAGGARGACAAGAC-3') and TEF-R (5'-GG-ARGTACCAGTSATCATGTT-3') primers were used for PCR, with an annealing temperature of 53°C (O'Donnell et al., 1998). Oligonucleotides were synthesized by Bioneer Corporation (Chungwon, Korea), dissolved at 100 μM in sterile water, and stored at 20°C. PCR product was purified using the Geneclean Turbo Kit (Qbiogene, Irvine, CA, USA) and cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) for sequencing. DNA sequencing was performed using an automated sequencer (ABI Prism 3700; Applied Biosystem, Foster, CA, USA) at the National Instrumentation Center for Environmental Management (Seoul National University, Seoul, Korea). The TEF gene sequences of KCTC16676 were used to compare with the TEF sequences archived in FUSARIUM-ID v.1.0 (http://fusarium.cbio.psu.edu; Geiser et al., 2004). Its sequences were aligned with TEF sequences of 23 *F. semitectum* and *F. equiseti* complexes with the CLUSTAL W program (Thompson et al., 1994) with default parameters. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with a Kimura-two parameter nucleotide substitution model in the MEGA3.1 program (Kumar et al., 2004). Bootstrap analyses were performed with 2000 replicates.

**Survey of apicidin production among *Fusarium* species.** The production of apicidin among isolates in the *F. semitectum-F. equiseti* species complex grown in the wheat culture was investigated using the protocol of Park et al. (1999) with a slight modification. A 20-g portion of each culture was extracted with 100 ml of chloroform for 30 min. After filtration through Whatman no. 2 filter paper, the filtrate was dried. The residue was dissolved in 2 ml of chloroform and applied to a Florisil column (2 cm [inside diameter] by 20 cm). The column was packed with 10 g of Florisil (60 to 100 mesh) topped with 5 g of anhydrous sodium sulfate. After washing with 100 ml of *n*-hexane, 100 ml of chloroform-methanol (3:1, vol/vol) was applied to the column for elution. After drying the eluate, it was redissolved in 2 ml of methanol and was analyzed using high-performance liquid chromatography (HPLC). For the HPLC analysis, a Shimadzu LC-10AD equipped with Shimadzu APD-10A detector (Shimadzu, Kyoto, Japan) was used. The column was a Symmetry $C_{18}$ column (4.6× 150 mm; Waters, Milford, MA, USA); UV=291 nm; the mobile phase was aqueous methanol; flow rate was 0.2 ml/min.

**Purification of apicidins.** Wheat cultures of KCTC16676 (4 kg) were extracted three times with chloroform (total of

17 liters), and the combined extracts were concentrated under reduced pressure. The concentrated chloroform extract was dissolved in 300 ml of distilled water and was defatted with 300 ml of *n*-hexane. The water fraction was extracted with 300 ml of chloroform, and the chloroform fraction was applied to a silica gel column (60×750 mm) and eluted with ethyl acetate-hexane (3:1, v/v). Each fraction was screened for apicidins by thin-layer chromatography (TLC), and fractions containing apicidin were combined and concentrated. TLC with pre-coated silica gel (Merck, Darmstadt, Germany) was used, and detection of apicidin was accomplished by spraying the plates with 5% ρ-anisaldehyde-sulfuric acid followed by heating as previously described (Park et al., 1999). The partially purified fraction was passed through silica gel (50×600 mm) followed by a reverse phase $C_{18}$ silica gel column (30×300 mm).

**Phytotoxicity of apicidins on plant seedlings.** Six plant species, cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon esculentum* Mill.), lettuce (*Lactuca sativa* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and soybean (*Glycine max* L.), were tested for their sensitivity to apicidin and apicidin $D_2$. Seeds were soaked in 1% NaClO for 10 min to eliminate seedborne fungi, and then washed with sterile distilled water for three times. Apicidin or apicidin $D_2$ was added to 2% water agar in glass test tubes autoclaved at 121°C for 20 min. Water agar without apicidins served as a negative control. Each seed was planted in the test tube sealed with a cotton plug. The tubes were placed in a growth chamber (Model EF7, Conviron, Winnipeg, Canada) for 14 days, set at a cycle of 14 h in light at 26°C and 10 h in dark at 18°C with 75% relative humidity. After 14 days, length and dry weight of plants (dried at 60°C for 2 days) were measured. This assay was performed twice with triplicates. The inhibition of plant growth by apicidin and apicidin $D_2$ was evaluated using the following formula: Growth inhibition (%) = $[(Growth_{control} - Growth_{test})/(Growth_{control})] \times 100$, where $Growth_{control}$=mean of growth of the control seedlings, $Growth_{test}$=mean of growth of the test seedlings. Minus sign indicates that growth of the seedling was not inhibited compared with the control. T test (LSD) was performed to evaluate phytotoxic effects of apicidin and apicidin $D_2$ with SAS statistical software version 6.04 (SAS Institute, Cary, NC, USA).

**Phytotoxicity of apicidins on weeds and vegetable plants.** Jimsonweed (*Datura stramonium* L.), wild spinach (*Chenopodium amaranticolor* L.), cucumber (*Cucumis sativus* L.) and tomato (*Lycopersicon esculentum* Mill.) were tested for sensitivity to apicidin and apicidin $D_2$. Seeds were planted individually in a commercial potting mixture supplemented with a slow release fertilizer (N-P-K, 14:14:14) and placed in a greenhouse. The greenhouse temperature was maintained at 28-32°C with 40-60% relative humidity. Two concentrations of apicidin and apicidin $D_2$ (10 and 20 μg/ml) dissolved in 1% methanol were used for the test. Two week-old plants (three to four-leaf stage including cotylendon) were sprayed with each toxin preparation (5 ml) using an aerosol sprayer. Distilled water containing 1% methanol was used as a negative control. After toxin application, plants were maintained for 14 days in the greenhouse and observed for phytotoxic symptoms such as chlorosis, necrosis, leaf rolling, stunting, and mortality during the observation period. After 14 days, height of the aboveground part was measured. This assay was performed twice with triplicates.

## Results and Discussion

**Identification of strain KCTC16676.** We sequenced a 667-bp TEF fragment amplified from KCTC16676, and searched FUSARIUM-ID v.1.0 (http://fusarium.cbio.psu.edu; Geiser et al., 2004) using the resulting sequence as a query. The TEF sequence of KCTC16676 showed high identity with those of the isolates in *F. pallidoroseum* (=*F. semitectum*; 90%), *F. scirpi* (91%) and *F. equiseti* (92-93%). We analyzed the phylogenetic position of KCTC16676 relative to 23 *F. semitectum* and *F. equiseti* isolates using the neighbor-joining method, which showed that KCTC16676 belongs to the *F. semitectum-F. equiseti* species complex (Fig. 2).

Although the *F. semitecum-F. equiseti* species complex has not yet been well delineated phylogenetically (D. Geiser, personal communication), two species can be differentiated morphologically. The shape of *F. semitectium* macroconidia is curved and tapering to a point at apical cells and is slender with a curved dorsal surface and a straighter ventral surface, while that of *F. equiseti* is elongated at apical cells and is curvature at both dorsal and ventral surface (Leslie and Summerell, 2006). The curved, pointed apical cells of KCTC16676 resembled those of *F. semitectum* and *F. sambucinum*, but other features, such as the rapid growth at 30°C and chemotype, are not typical of *F. sambucinum* (Park et al., 1999). Based on both of the phylogenetic and morphological characteristics, we tentatively identified that the strain is *F. semitectum*.

**Enhancement of apicidin production by KCTC16676 strain.** Air-dried wheat cultures of KCTC16676 strain were extracted with chloroform. Through silica gel and reverse-phase $C_{18}$ column, apicidin and two known analogs, apicidin A and apicidin $D_2$, were purified. We purified 5 g of apicidin, 200 mg of apicidin A, and 300 mg of apicidin $D_2$

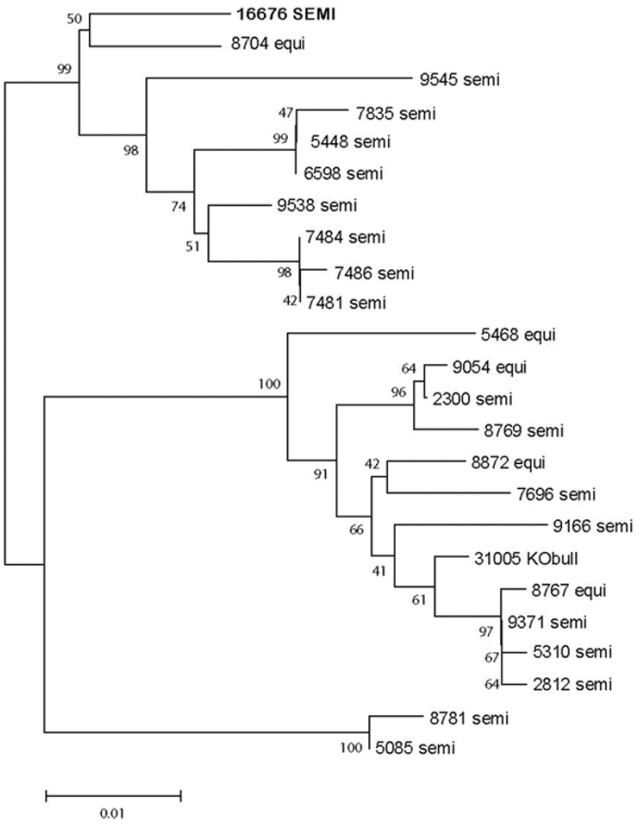


**Fig. 2.** Dendrogram showing the phylogenetic framework based on the TEF gene sequences of strain KCTC16676 and 23 isolates belonging to the *F. semitectum-F. equiseti* species complex. A phylogenetic tree was constructed using the neighbor-joining method with a Kimura-two parameter nucleotide substitution model in the MEGA3.1 program (Kumar et al., 2004). Bootstrap analyses were performed with 2000 replicates. 16676SEMI indicates KCTC16676; semi indicates *F. semitectum* strains; equi indicates *F. equiseti* strains; KObull indicates *F. semitectum* strain.

from 4 kg of wheat cultures.

We previously purified apicidin from the ethyl acetate extract of wheat culture of KCTC16676 through normal phase columns, Florisil and Silica gel (Park et al., 1999). In this study, we used chloroform instead of ethyl acetate for apicidin extraction because the chloroform extract contained less pigments than the ethyl acetate extract. The average amount of apicidin purified from wheat cultures was 0.3 mg/g in the previous study, whereas in this study, it was 1.3 mg/g. We also found that using the reverse $C_{18}$ column was efficient to remove the pigments in the culture extracts and to separate apicidin from its anlalogs, apicidin A and apicidin $D_2$.

The production of apicidin is not common amongst *Fusarium* spp. We previously screened for apicidin production among 52 isolates from 20 soybean samples, representing 7 *Fusarium* spp. and found only two apicidin-producing strains, KCTC16676 and 16677 (Park et al., 1999). We checked for apicidin production among additional 23 isolates belonging to the *F. semitectum-F. equseti* complex in this study, but no strains produced apicidin, suggesting that the ability to produce apicidin is not widely distributed among *Fusarium* spp., even among isolates in the *F. semitectum-F. equseti* complex.

**Phytotoxicity of apicidins on plant seedlings.** At 5 μg/ml apicidin, seedlings of most plants were not visibly inhibited. Only root length of maize and root dry weight of lettuce were significantly affected (Table 1). At 10 μg/ml of apicidin, the length and dry weight of shoot in all plants were significantly reduced. Root dry weight of all plants

**Table 1.** Effects of apicidin and apicidin $D_2$ on seedling growth of six plant species

| | Apicidin (μg/ml) | | | Apicidin $D_2$ (μg/ml) | | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 20 | 5 | 10 | 20 |
| Shoot length (% of growth inhibition)* | | | | | | |
| Maize | 5 a† | 23 b | 70 ab | –38 c | 3 bc | 54 b |
| Cucumber | –2 a | 51 a | 58 c | –16 b | –8 c | 16 c |
| Lettuce | 21 a | 21 b | 51 c | 25 a | 40 a | 55 b |
| Soybean | 8 a | 25 ab | 74 a | –3 b | 26 ab | 78 a |
| Tomato | 3 a | 43 ab | 73 a | –4 b | 33 a | 56 b |
| Wheat | 11 a | 27 ab | 61 bc | 11 a | 22 ab | 55 b |
| Shoot dry weight (% of growth inhibition) | | | | | | |
| Maize | 22 a | 22 a | 86 a | –12 bc | 27 a | 79 a |
| Cucumber | –8 a | 27 a | 34 c | –28 c | –9 b | 24 c |
| Lettuce | 16 a | 16 a | 37 c | 16 a | 26 a | 37 bc |
| Soybean | 18 a | 14 a | 31 c | 9 ab | 17 ab | 32 bc |
| Tomato | 8 a | 32 a | 68 b | 0 ab | 12 ab | 32 bc |
| Wheat | 6 a | 19a | 65 b | 6 ab | 15 ab | 53 ab |
| Root length (% of growth inhibition) | | | | | | |
| Maize | 32 a | 56 ab | 86 a | –15 c | 43 a | 80 a |
| Cucumber | –14 b | 8 c | 1 c | –22 c | –15 b | –15 c |
| Lettuce | 20 ab | 25 c | 45 b | 32 a | 35 a | 47 c |
| Soybean | 15 ab | 19 c | 56 b | 5 b | 27 a | 59 b |
| Tomato | –2 ab | 43 bc | 51 b | 2 bc | 30 a | 42 c |
| Wheat | 22 ab | 72 a | 84 a | –5 b | 35 a | 49 c |
| Root dry weight (% of growth inhibition) | | | | | | |
| Maize | 20 ab | 34 b | 78 b | –12 ab | 18 ab | 70 b |
| Cucumber | –9 b | 32 b | 54 c | –20 b | –7 b | 47 c |
| Lettuce | 40 a | 33 b | 60 c | 20 a | 47 a | 67 b |
| Soybean | 30 ab | 73 a | 94 a | 2 ab | 63 a | 94 a |
| Tomato | –8 b | 46 ab | 62 c | 0 ab | 31 ab | 54 bc |
| Wheat | 13 b | 45 ab | 67 bc | –17 b | 26 ab | 64 b |

*$[(Growth_{control} - Growth_{test})/(Growth_{control})] \times 100$, where $Growth_{control}$ = mean of growth of the control seedlings, $Growth_{test}$ = mean of growth of the test seedlings. Minus sign indicates that growth of the seedling was not inhibited compared with the control.

†Means of duplicates. Values within a column not sharing a letter are significantly different according to T-test (LSD, P=0.05).

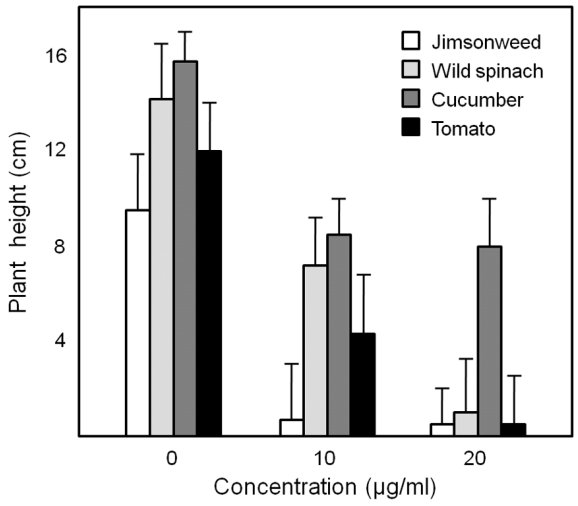


**Fig. 3.** Growth inhibition of weeds and vegetable plants by apicidin. Apicidin dissolved in 1% methanol was applied on 2-week-old plants, and plant height was measured 14 days after treatment.

was significantly affected by apicidin but root length of all plants except for maize looked comparable to that of the negative control. At this concentration, negative effect of apicidin $D_2$ was weaker than that of apicidin in most cases (Table 1). At 20 μg/ml, all plants and traits, except the root length of cucumber, were significantly affected by apicidins (Table 1). Although the root length was less affected by apicidins, root development such as root hair and spreading of roots were severely inhibited (data not shown). The phytotoxicity of apicidin was weaker than that of T-2 toxin, DON, 15-ADON and HC-toxin, which exhibited phytotoxic activity at concentration 1-10 μg/ml and inhibited root growth severely (Abbas et al., 2001; Desjardins, 2006).

**Phytotoxicity of apicidins on weeds and vegetables.** At 10 μg/ml, phytotoxic symptoms appeared in all tested plants within 1-2 days after treatment with apicidins. The symptoms included chlorosis and necrosis on leaves, distortion of leaf shape, and growth inhibition (data not shown). Especially in tomato and jimsonweed, a severe damage occurred in the basal part of stems followed by rotting, and plants were fallen down to the ground. However, other plants recovered from the damages within a week. In cucumber, chlorosis, necrosis, leaf rolling, and stunting occurred on older leaves within 2 days after treatment. Leaf shape distortion was observed on immature leaves, but no mortality was observed in cucumber. The heights of wild spinach, cucumber, and tomato were greatly reduced as compared with the control (Fig. 3). Apicidin $D_2$ also caused similar damages, but its phytotoxicity was less than that of apicidin (data not shown). At 20 μg/ml, only cucumber survived after treatment, but the height of cucumber was severely reduced. Jimsonweed, wild spinach, and tomato were completely destroyed. Apicidin $D_2$ showed a similar pattern of phytotoxic damages on these plants, but took a longer period to cause those damages (data not shown).

In conclusions, we determined that an apicidin-producing strain KCTC16676 belongs to the *F. semitectum*, improved the extraction procedure to enhance the yield and purify of apicidin, and showed phytotoxicity of apicidins. In many cases, genes required for the secondary metabolites of fungi including *Fusarium* spp. are clustered in the genome, and the expression of those genes is controlled by transcription factor(s) that are also located in the cluster (Lee et al., 2001; Kim et al., 2005). We hypothesize that the genes involved in apicidin biosynthesis are clustered. Attempts to find such genes are in progress through the use of both forward and reverse genetics approaches. Identification of transcription factor(s) controlling apicidin biosynthesis genes will help us overproduce apicidin by upregulating the downstream genes in KCTC16676.

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