Biometals (2010) 23:1053–1060
DOI 10.1007/s10534-010-9350-0

# Cu(II): a "signaling molecule" of the mangrove endophyte *Fusarium oxysporum* ZZF51?

**Jia-hui Pan · Yong-cheng Lin · Ni Tan · Yu-cheng Gu**

Received: 3 February 2010 / Accepted: 23 May 2010 / Published online: 6 June 2010


**Abstract** We previously reported the isolation of Cu–fusaric acid (Cu–FA) complex from the mangrove endophyte *Fusarium oxysporum* ZZF51. In this study, we explored the mechanism of Cu–FA production in the strain ZZF51 by comparing with that of another endophyte *Fusarium* sp. B2, which produced FA but not Cu–FA in the same culture condition. The results allowed us to hypothesize that $Cu^{2+}$ may act as a "signaling molecule" to awaken the silent FA biosynthetic genes in ZZF51, inducing intracellular production of FA followed by chelation with $Cu^{2+}$. This signaling network was triggered specifically by $Cu^{2+}$ and may be interfered by other metal ions.

**Keywords** Copper–fusaric acid · *Fusarium* sp. · Signaling molecule

J. Pan · Y. Lin (✉) · N. Tan
School of Chemistry and Chemical Engineering, Sun Yat-sen University, 135 Xingang West Road, 510275 Guangzhou, China
e-mail: ceslyc@mail.sysu.edu.cn

Y. Gu
Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK

## Introduction

Among the characteristics of filamentous fungi, the regulation of secondary metabolites biosynthesis renders them of great interest to the research community. Besides the regulation within the biosynthetic cluster, external control of the cluster, and global regulation of secondary metabolism, many environmental factors control the biosynthesis of these natural products, including nutrient conditions, temperature, light, pH, and metals (Fox and Howlett 2008; Shwab and Keller 2008). Metal availability appears critical to the biosynthesis of some fungal secondary metabolites, with mytotoxins being the best documented class. $Zn^{2+}$ is specifically required in the biosynthesis of fusaric acid in *Fusarium vasinfectum* (Kalyanasundaram and Sarasvati-Devi 1955) and aflatoxin in *Aspergillus parasiticus* (Mateles and Adye 1965). Maintaining endogenous $Ca^{2+}$ homeostasis is required for cercosporin biosynthesis in *Cercospora nicotianae* (Chung 2003). $Mn^{2+}$ is shown to be a crucial factor regulating the onset of 3-acetyldeoxynivalenol biosynthesis by *Fusarium graminearum* (Vasavada and Hsieh 1988). However, the pivotal effects of metal-signaling on the biosynthesis of these metabolites have never been addressed at the molecular level, until Cuero et al. (2003) explored the molecular significance of metals on production of aflatoxin.

Cu–fusaric acid complex (Cu–FA), with the chemical name of bis(5-butyl-2- pyridinecarboxylatoN1,O2)-copper (Fig. 1), was previously isolated

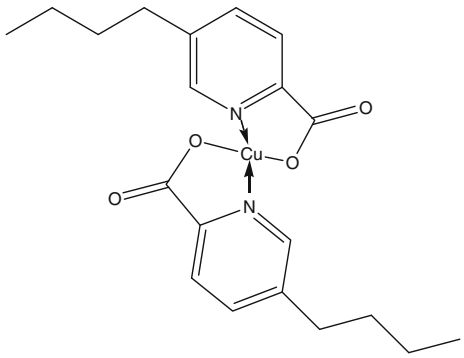


**Fig. 1** Structure of Cu–fusaric acid (Cu–FA)

from a mangrove endophytic fungus *Fusarium oxysporum* ZZF51 by our group (Tan et al. 2008). From another endophyte *Fusarium* sp. B2 (Zhang et al. 2009) collected from the mangrove in the same sea area, only FA but no Cu–FA was isolated in the same culture condition. This phenomenon attracted our attention and great interest. By the parallel experiments with ZZF51 and B2, we explored how Cu–FA and FA were produced in these strains. We also tested if the production of FA–metal complex was specifically stimulated by $Cu^{2+}$, or if other metal ions would function in the same way.

## Materials and methods

### Source and identification of fungi

*F. oxysporum* ZZF51 and *Fusarium* sp. B2 were isolated from the leaf of *Castanopsis fissa* and the seeds of *Kandelia candel*, respectively, from Zhanjiang sea area, China. ZZF51 was identified based on combined DNA sequences from the internal transcribed spacer (ITS) regions and the translation elongation factor 1α (EF-1α) (Ignazio and Linda 1999) (Genebank accession numbers: EU219559 and FJ387167). B2 was identified based on the morphological characteristics growing on potato dextrose agar (PDA) and synthetic low-nutrient agar (SNA) medium (Nirenberg and O'Donnell 1998).

### Fermentation

Mycelial agar plugs were inoculated into 500 ml Erlenmeyer flasks containing 300 ml GPY broth (1% glucose, 0.2% peptone, 0.1% yeast extract, and 0.4% sea salt). The series of modified media (pH 7.0) were prepared with the final $CuCl_2{\cdot}2H_2O$ concentration to be 10, 20, 50, 100, 200 and 400 mg/l, respectively. The strains of ZZF51 and B2 were incubated at 28°C for 25 days under stationary conditions. A positive control (no addition of $CuCl_2{\cdot}2H_2O$ but with fungal inoculation) was used.

### Copper distribution in mycelia

Copper distribution in mycelia was determined by a sequential elution procedure (Taboski et al. 2005). The method recovered the copper content from both extracellular (Cu bound to the polar groups on the outer cell wall surface) and intracellular (Cu transported into the cells) mycelium. After harvest, the mycelia were eluted thoroughly with ultra-pure water. To remove extracellular Cu elements, the mycelia were washed with four 50 ml portions of 100 mM EDTA (pH 4.5) for 30 min each time. The washings were collected by filtration and treated with concentrated and 1.0 M $HNO_3$ consecutively as described below. The remaining mycelial samples were dried by freeze dryer to record their dry weights. A sample of each dry mycelium was taken and treated with concentrated $HNO_3$, followed by dilution with 1.0 M $HNO_3$ to a target volume for Cu analysis. Cu content from both extracellular and intracellular were subsequently analyzed using a Model Z-2000 series Polarized Zeeman Atomic Absorption Spectrometer (Hitachi).

### Quantification and qualification of Cu–FA and FA in fermentation broths

After the fermentation processes, the cultures were filtered. The filtrate (300 ml) was extracted with 500 ml of ethyl acetate (EtOAc) for three times. The combined EtOAc solution was evaporated and dried under vacuum; the residues were dissolved in methanol, prepared for qualification of Cu–FA and FA. HPLC was performed on a LC-20A (Shimadzu) system equipped with a SPD-M20AV PDA detector and a Ultimate XB-C18 column (4.6 × 250 mm, 5 μm) (Welch Materials), eluted with 80% methanol and 20% of ultra-pure water at a flow rate of 0.9 ml/min. The detection for Cu-FA and FA were carried at

269 nm (Tan et al. 2008; Amalfitano et al. 2002). LC/MS data were acquired using a LCQ DECA XP (Thremo) system with the same column and elution condition as mentioned above.

### Test of copper specificity

The GYP medium was independently amended with varied metal ions before inoculation, at concentrations of $ZnCl_2$ (20, 40 and 60 mM), $CdCl_2{\cdot}2.5H_2O$ (0.6, 1.2 and 1.8 mM) and $CoCl_2{\cdot}6H_2O$ (0.5, 2, and 4 mM) in single form. Combinative treatments were prepared in duplex (10 mg/l $CuCl_2{\cdot}2H_2O$ + 40 mM $ZnCl_2$, 10 mg/l $CuCl_2{\cdot}2H_2O$ + 1.2 mM $CdCl_2{\cdot}2.5H_2O$, 10 mg/l $CuCl_2{\cdot}2H_2O$ + 2 mM $CoCl_2{\cdot}6H_2O$). After harvest, the EtOAc extracts of culture supernatant were prepared as described previously for HPLC and LC/MS analysis. Co–FA, Zn–FA and Cd–FA were synthesized and characterized by Dr. Yi-wen Tao (School of Chemistry and Chemical Engineering, Sun Yat-sen University). The detection of Co/Zn/Cd–FA in metabolites was performed on HPLC (detected in 269 nm) with authentic standards of these three complexes and further confirmed by LC/MS.

## Results

### Effect of copper on fungal growth and morphological changes

Exposure of ZZF51 to increasing $Cu^{2+}$ concentrations resulted in color changes and morphological differences. The colonies in the control medium was purplish, whereas the colonies at the highest $Cu^{2+}$ treatment (400 mg/l $CuCl_2{\cdot}2H_2O$) were greenish. Increasing concentrations of $Cu^{2+}$ also resulted in typically abnormal fungal morphological changes under environmental stresses (TE2000 light microscopy, Nikon) i.e. the amounts of chlamydospores markedly increased, and the conidial germination, especially for macroconidia was significantly depressed (data not shown). Treatments ranging from 0 to 200 mg/l did not display a regular growth pattern, although exposure to 10 and 20 mg/l of $CuCl_2{\cdot}2H_2O$ markedly stimulated growth compared with controls. Significant depression ($P < 0.001$) on growth for the most rigorous treatment (400 mg/l) was expected (Fig. 2).

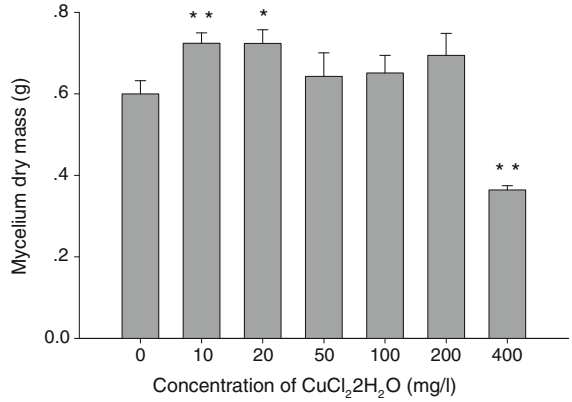


**Fig. 2** Effect of $Cu^{2+}$ concentrations on growth (mg dry wt/300-ml fermentation broths) of ZZF51 in stationary liquid media (25 days) amended with increasing concentrations of $CuCl_2{\cdot}2H_2O$ from 0 to 400 mg/l. Data represent means ± SD of three replicates. *Single* and *double asterisks* indicate significant ($P < 0.05$) and highly significant ($P < 0.001$) differences, respectively

### Copper in the mycelia

Except for the data of the 400 mg/l treatment of $CuCl_2{\cdot}2H_2O$, the copper concentrations from both extracellular and intracellular samples of ZZF51 (mg of Cu/g of mycelium) increased with increasing $CuCl_2{\cdot}2H_2O$ concentrations in the media, with the correlation coefficients of 0.988 and 0.958, respectively (Fig. 3). Cu was detected in both mycelial compartments with an uneven distribution. The majority of the Cu was present in the intracellular fraction, which contained a declining percentage of the total Cu in the mycelia from 94.2 to 58.9% when the concentration of $CuCl_2{\cdot}2H_2O$ increased from 0 to 200 mg/l. As previously shown in Fig. 2, growth performance of the strain ZZF51 was significantly depressed with the concentration of $CuCl_2{\cdot}2H_2O$ at 400 mg/l, which may be accompanied by a depression in the function of Cu transporting system. As a result, the intracellular copper concentration was sharply decreased (Fig. 3).

### Copper in the metabolites

The total Cu from the control GYP medium was determined to be 0.029 ± 0.002 (mg Cu/300 ml broth) by inductively coupled plasma-atomic emission spectrometer (ICP, TJA IRIS HR). In this control

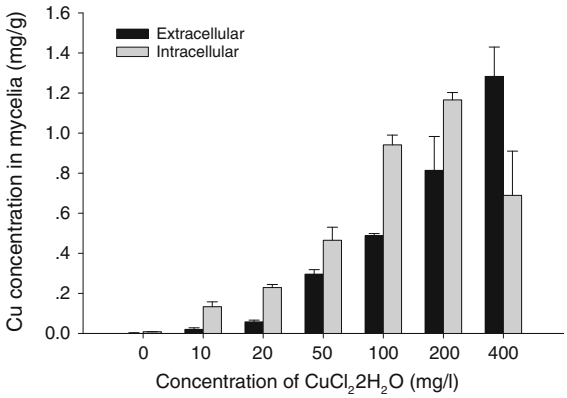


**Fig. 3** Cellular location of Cu in ZZF51 (25 days, in stationary liquid media amended with increasing concentrations of $CuCl_2{\cdot}2H_2O$ from 0 to 400 mg/l). Data were expressed as mg of Cu in 1 g of mycelium. Data represent means ± SD of three replicates. Data at all six Cu concentrations are significant ($P < 0.05$) compared with control

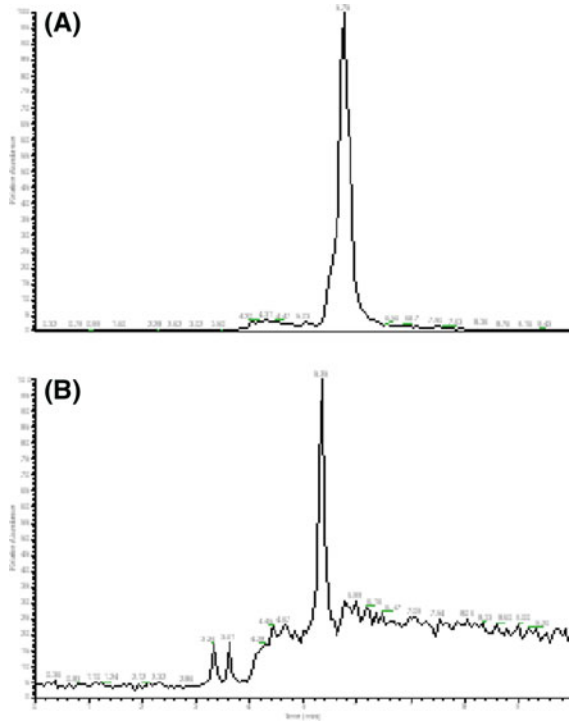


**Fig. 4** LC/MS (SIM mode, selected ion monitoring) traces of the extracts of control cultures (no supplements of $Cu^{2+}$) of ZZF51 (**a**) and B2 (**b**) (25 days, stationary cultures), with center mass aiming at both 420 ($[M_{Cu-FA} + H]^+$/width = 1.00) and 180 ($[M_{FA} + H]^+$/width = 1.00)

medium, Cu–FA can be found in the fermentation broth of ZZF51, whereas free FA was undetectable (Fig. 4a). HPLC analysis showed that ZZF51 yielded an increasing production of Cu–FA with the concentration of $CuCl_2{\cdot}2H_2O$ increasing from 0 to 20 mg/l (0.097 ± 0.024 for the control and 0.182 ± 0.038 for 20 mg/l treatment) (Table 1). Within this range of treatment, a strong correlation (r = 0.9979) was also observed between intracellular copper concentrations and the yields of Cu–FA, suggesting that the production of Cu–FA is highly dependent on the amount of copper transporting into the cells. The yield however sharply decreased to ca. 55% of control for 50 mg/l treatment (0.054 ± 0.013) and no Cu–FA can be found any more in 100 mg/l or higher copper treatments (Table 1). Of note, FA was undetectable by LC/MS analysis throughout from the control to the highest copper treatments. Meanwhile the production of Cu–FA by ZZF51 was also observed at different harvest time (Table 1). It was found that in the control cultures the yields of Cu–FA increased markedly over time (0.036 ± 0.008 for 15 days and 0.097 ± 0.024 for 25 days), whereas for the 10 mg/l treatment it remained virtually unchanged after 15 day (0.138 ± 0.030 for 15 days and 0.140 ± 0.032 for 25 days).

To further understand the mechanism underlying the response of ZZF51 to $Cu^{2+}$, extracts of fermentation broths of *Fusarium* sp. B2, another mangrove endophyte capable of producing FA but not Cu–FA (Zhang et al. 2009), was analyzed as a parallel comparison. In contrast with ZZF51, FA can be found in the culture broth of B2 throughout the treatments from the control to 400 mg/l $CuCl_2{\cdot}2H_2O$. No Cu-FA was detected in the control cultures of B2 (Fig. 4b), while exposure to 10–400 mg/l $CuCl_2{\cdot}2H_2O$ treatments yielded a similar pattern of Cu-FA production with that of ZZF51 (Table 1).

## Copper specificity

In order to test if the production of FA–metal complex was specifically stimulated by $Cu^{2+}$, or if there are interactions between $Cu^{2+}$ and other metal ions influencing the biosynthesis of Cu–FA, varied concentrations of metal ions were added into the GYP medium singly or in duplex. All the treatments used in this study showed no significant effect on fungal growth ($P < 0.05$ compared with the control) or morphological changes of ZZF51. Chloride salts was

**Table 1** Effect of $Cu^{2+}$ concentrations on production of Cu–FA (stationary cultures)

| Concentration of $CuCl_2 \cdot 2H_2O$ (mg/l) | Production of Cu–FA (mg/300-ml fermentation broths) | | |
|---|---|---|---|
| | ZZF51 | | B2 |
| | 15 days | 25 days | 25 days |
| 0 | 0.036 ± 0.008 | 0.097 ± 0.024 | –[a] |
| 10 | 0.138 ± 0.030 | 0.140 ± 0.032 | 0.101 ± 0.020 |
| 20 | n.d. | 0.182 ± 0.038 | 0.203 ± 0.043 |
| 50 | n.d. | 0.054 ± 0.013 | 0.076 ± 0.021 |
| 100 | n.d. | –[a] | –[a] |
| 200 | n.d. | –[a] | –[a] |
| 400 | n.d. | –[a] | –[a] |

*n.d.* not determined

[a] Cu–FA was undetectable

used to keep consistency. Results showed that in the medium without supplement of $Cu^{2+}$, the externally added $Zn^{2+}$, $Co^{2+}$ and $Cd^{2+}$ did not result in the production of Zn–FA/Co–FA/Cd–FA, meanwhile a total inhibition of Cu–FA production was observed (Fig. 5, upper). LC-MS analysis confirmed this result and further showed that no FA was detected in all these treatments either (data not shown). In combinative metal treatments ($Cu^{2+}$–$Co^{2+}$, $Cu^{2+}$–$Zn^{2+}$, and $Cu^{2+}$–$Cd^{2+}$), Zn–FA/Co–FA/Cd–FA remained undetectable in LC-MS analysis (data not shown). Cu–FA can be found but its production was significantly lower ($P < 0.01$) compared to that cultured in the single $Cu^{2+}$ treatment (Fig. 5, lower).

## Discussion

The role of metals in the regulation of secondary metabolism in microorganisms is well documented. Metals function as cofactors of crucial enzymes, or interact with operator genes that control formation of secondary metabolic synthases or proteins. They are also important in modulating cell wall permeability and in regulating many pre- and posttranscriptional events (Rutherford and Bird 2004). Since Cu-FA was isolated as a secondary metabolite from *Fusarium oxysporum* ZZF51, its biosynthesis has attracted our attention and great interest. In this study we aimed to explore the mechanisms underlying its production in ZZF51 by comparing with that of another endophyte *Fusarium* sp. B2.

Results showed that in the control medium, where a minute amount of copper exists (0.029 ± 0.002 mg/300-ml broth), only Cu–FA but no FA was produced by ZZF51, whereas only FA but no Cu-FA was produced by B2 (Fig. 4). For $Cu(FA)_2$ $Ksp = [Cu^{2+}(aq)] * [FA^{-}(aq)]^2$ at equilibrium. If $[Cu^{2+}(aq)] * [FA^{-}(aq)]^2 > Ksp$, a precipitate of $Cu(FA)_2$ will form; otherwise the precipitate will not form. In the same control medium, $[Cu^{2+}(aq)]$ are the same for both ZZF51 and B2, but $[FA^{-}(aq)]_{B2}$ was obviously larger than $[FA^{-}(aq)]_{ZZF51}$ because FA was a metabolite of B2 as previously reported. Therefore, if no Cu–FA was formed in the control culture of B2, i.e. $[Cu^{2+}(aq)] * [FA^{-}(aq)]^2 < Ksp$, normally Cu–FA should not have been formed in ZZF51 either. However, the present results do not appeared to follow this rule. An exceptional condition was therefore hypothesized as follows: ZZF51 should not have produced FA as a metabolite, with the involved biosynthetic gene cluster switched off, or at least down-regulated; the minute amount of $Cu^{2+}$ transported into the cells acted as a "signaling molecule" inducing the intracellular production of FA, followed by the chelation with the intracellular $Cu^{2+}$ to produce Cu–FA. Apart from co-cultivation and genomic approaches, challenging microorganisms through culture conditions and external cues have been commonly known as an effective way of triggering "cryptic" biosynthetic pathways to yield "cryptic natural products" (Scherlach and Hertweck 2009). Heavy metal ions, as one of the most important nutritional and environmental factors, may not

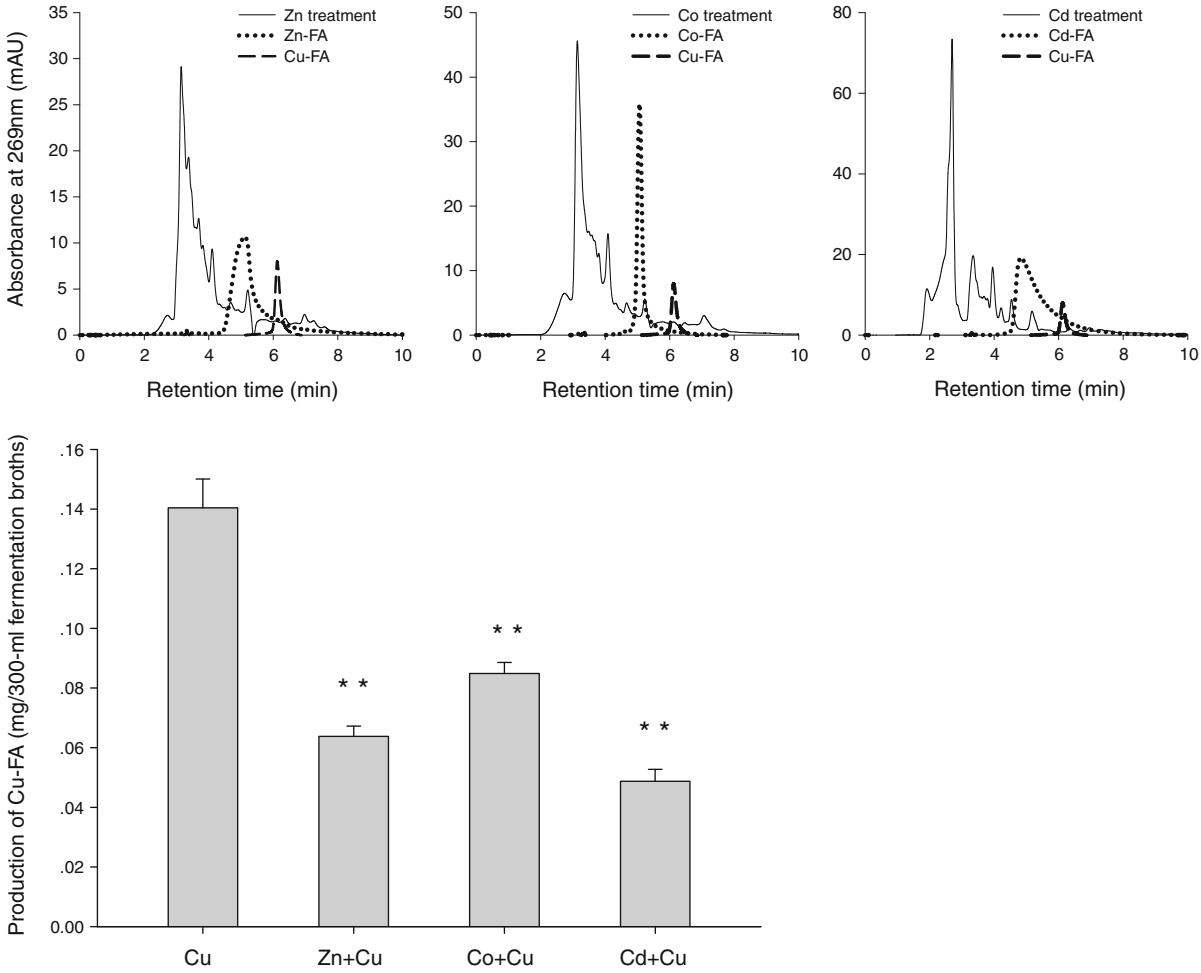


**Fig. 5** (*Upper*) HPLC graphs (detected at 269 nm) of the extracts of ZZF51 broths under Zn, Co and Cd treatments in a single form (no supplement of Cu). Standards of Zn–FA/Co–FA/Cd–FA and Cu–FA were run in parallel. Zn–FA and Cd–FA exhibited broad peaks in HPLC analysis under a variety of eluting conditions, but it was acceptable from a screening purpose. All the results were confirmed by LC-MS analysis. (*Lower*) Production of Cu–FA (mg/300-ml fermentation broths) in the presence of a combination of 10 mg/l $CuCl_2{\cdot}2H_2O$ + 40 mM $ZnCl_2$, 10 mg/l $CuCl_2{\cdot}2H_2O$ + 1.2 mM $CdCl_2{\cdot}2.5H_2O$, 10 mg/l $CuCl_2{\cdot}2H_2O$ + 2 mM $CoCl_2{\cdot}6H_2O$. Data at all combinative treatments are highly significant ($P < 0.01$, indicated by *double asterisks*) compared with the single $Cu^{2+}$ treatment (10 mg/l $CuCl_2{\cdot}2H_2O$)

only impact the quantity of a certain compound but also the general metabolic profile of an organism (Haferburg et al. 2009). Therefore, we hypothesized that $Cu^{2+}$ function as an external cue awakening the concerted FA biosynthetic genes in ZZF51. This however raised another question: if Cu–FA was secreted out of the cells in a usual way, why didn't it dissociate with such low concentrations of $Cu^{2+}$and $FA^{-}$? Therefore we proposed a bold yet plausible hypothesis that could account for the observations: the Cu–FA might be enwrapped by certain lipophilic metabolites and then transported out of cells as an enwrapped form, preventing the dissociation of Cu–FA. When the culture broth was extracted by EtOAc, the lipophilic 'coat' was unwrapped, and Cu–FA was extracted and isolated in its original form. Further experiments should be conducted to back up this explanation, but once this hypothesis was testified, the phenomenon would be very interesting. On the other hand, FA was an extracellular metabolite of B2. The concentration of FA in the culture broth of B2 was relatively high, but

$[Cu^{2+}(aq)]_{B2} * [FA^{-}(aq)]^{2}_{B2}$ was still lower than Ksp due to the low concentration of $Cu^{2+}$ in the control medium Therefore, Cu–FA cannot be formed.

When the concentration of $CuCl_2{\cdot}2H_2O$ increased from 10 to 50 mg/l, the pattern of Cu–FA production was similar for both two strains, i.e. the yield of Cu–FA increased with increasing addition of $CuCl_2{\cdot}2H_2O$ from 10 to 20 mg/l, followed by a decrease between 20 to 50 mg/l $CuCl_2{\cdot}2H_2O$ (Table 1). It is noteworthy that FA was undetectable throughout in the culture broth of ZZF51 under all the treatments, whereas it can be found throughout in that of B2. Therefore, we assumed that the two strains adopted different mechanisms to produce Cu–FA. For ZZF51, a strong correlation ($r = 0.9979$) between intracellular copper concentration and the yield of Cu–FA was also observed for the treatments with the $CuCl_2{\cdot}2H_2O$ concentrations increasing from 0 to 20 mg/l, suggesting that the production of Cu–FA (*cellular response*) is highly dependent on the amount of copper transporting into the cells (*external signals*, no matter bypassing the plasma membrane or not). This indicated that once the biosynthetic genes of FA were awakened, the accumulated intracellular copper may result in an increased expression of relevant regulatory proteins, leading to an increased amount of FA. This is a process of stimulation and response, so there was no excess of FA detectable throughout. This hypothesis subsequently raises a series of interesting and fundamental questions: can $Cu^{2+}$ bypass the plasma membrane and penetrate directly into cells to initiate the intracellular signaling chain? Otherwise what is the plasma membrane copper sensor and how copper recognize it? Obviously a considerable amount of work has to be done to test the hypothesis in a molecular level. On the other hand, we surmised that the production of Cu–FA by B2 was probably attributed to the mechanism of metabolic equilibrium. Continuous addition of $Cu^{2+}$ reached the concentration that Ksp required, leading to the formation of Cu–FA and ending up with a reduction of 'free' FA molecules. This elicited a positive feedback, leading to a continuous extracellular production of FA to maintain its concentration at a certain level. Therefore, FA can be found throughout, and Cu–FA is likely not a 'secondary metabolite' of B2 but rather a product of chelation in the culture broth.

Higher concentrations of $Cu^{2+}$, i.e. 100 mg/l $CuCl_2{\cdot}2H_2O$ and above, as an overwhelming metal stress, presumably destroyed their metabolic process. Therefore, neither FA nor Cu–FA was detected in the culture broth of both strains.

We were curious about the fact that when cultured in the control medium, only Cu–FA but no other FA–metal complexes were isolated from ZZF51. Thus, the possibility that $Cu^{2+}$ may act as a specific signal had been considered. Results showed that when the culture medium was amended with the individual $Co^{2+}/Zn^{2+}/Cd^{2+}$ ion, neither Co–FA, Zn–FA, Cd–FA, Cu–FA nor FA was detected as a metabolite in the culture broth. It suggested that (1) $Cu^{2+}$ was a specific trigger of the cryptic FA biosynthesis genes in ZZF51, while other metal ions cannot function in the same way, and (2) other ions negatively influencing the $Cu^{2+}$ signaling network, i.e. $Cu^{2+}$ cannot successfully switch on the silent FA genes in the presence of other metal ions. In a further study we explored if there are interactions between $Cu^{2+}$ and other metal ions influencing the biosynthesis of Cu–FA. It turned out that combinative metal treatment, i.e. a duplex of $Cu^{2+}$–$Co^{2+}$, $Cu^{2+}$–$Zn^{2+}$, and $Cu^{2+}$–$Cd^{2+}$ yielded a significantly lower ($P < 0.01$) production of Cu–FA compared with that in the single $Cu^{2+}$ treatment (Fig. 5, lower). The suppressed production of Cu–FA further demonstrated that the other metal ions interfered with the induction of FA biosynthetic pathway triggered by $Cu^{2+}$.

We also studied the speed of Cu–FA production by ZZF51, and found that it varied in the presence of different $Cu^{2+}$ concentrations. In the control medium (no supplement of $Cu^{2+}$) the production of Cu–FA increased markedly over time, whereas for the 10 mg/l treatment the yield remained virtually constant after 15 day (Table 1). Although the mechanism accounting for this difference is hitherto unknown, we assumed that the control medium resembled a typical marine ecosystem where trace amounts of copper exist, and the fungus adjusted their metabolic pathway to the environment gradually as they grow over time. Thus it results in a slow initiation of the signaling chain and then a slow increase in Cu–FA production. The addition of $CuCl_2{\cdot}2H_2O$ into the medium functioned as a strong signal, stimulating the up-regulation of FA production pathways and subsequently accelerating the production of Cu–FA.

To reveal the molecular mechanism of Cu-FA production and the $Cu^{2+}$ function in ZZF51, a crucial problem remained to be solved is the biosynthesis

pathway of FA. Research on *Fusarium* mycotoxin biosynthesis has gained significant progress for the last decade; seven classes of mycotoxin biosynthetic genes or gene clusters have been identified so far (Desjardins and Proctor 2007). However, FA as a structurally simple *Fusarium* mytotoxin, its biosynthetic genes still remain unknown, so obviously a large amount of difficult work has to be done. Preliminary studies were only conducted by radioactive tracing, and the results indicated that FA is formed from a polyacetate unit and aspartate or closely related metabolites (Hill et al. 1966; Dobson et al. 1967; Stipanovic et al. 2008), suggesting that FA is a putative polyketide. Based on this information and the recent progress on activation of fungal silent gene clusters (Brakhage et al. 2009), we are currently using genomics techniques, focusing our efforts towards the ultimate goal: reveal the molecular mechanism of Cu–FA production and the $Cu^{2+}$ function in ZZF51. In conclusion, our evidence to date suggests that $Cu^{2+}$ may act as a "signaling molecule" to awaken the silent FA biosynthetic genes in ZZF51, inducing intracellular production of FA followed by chelation with $Cu^{2+}$. This signaling network was triggered specifically by $Cu^{2+}$ and may be negatively influenced by other metal ions. Once these hypotheses are justified, we believe it will provide novel and significant mechanistic insight into a microbial cellular function responsive to external cues.

**Acknowledgements** This research was supported by the National Natural Science Funds of China (20772162, 20572136), the 863 Funds of China (2007AA09Z448, 2006AA09Z422), and the Science Funds of Guangdong Province, China (2007A032600001). We also appreciate the PhD studentship awarded to Jia-hui Pan by Syngenta Ltd. Helpful discussions with Dr. Yi-wen Tao and Zhong-shan Cheng are gratefully acknowledged.

## References

Amalfitano C, Pengue R, Andolfi A, Vurro M, Zonno MC, Evidente A (2002) HPLC analysis of fusaric acid, 9,10-dehydrofusaric acid and their methyl esters. Toxic metabolites from weed pathogenic *Fusarium* species. Phytochem Anal 13:277–282

Brakhage AA, Bergmann S, Schuemann J, Scherlach K, Schroeckh V, Hertweck C (2009) Fungal genome mining and activation of silent gene clusters. In: Anke T, Weber D (eds) Physiology and Genetics. The Mycota XV, 1st edn. Springer, Berlin, Heidelberg, New York, pp 297–303

Chung KR (2003) Involvement of calcium/calmodulin signalling in cercosporin toxin biosynthesis by *Cercospora nicotianae*. Appl Environ Microbiol 69:1187–1196

Cuero R, Ouellet T, Yu J, Mogongwa N (2003) Metal ion enhancement of fungal growth, gene expression and aflatoxin synthesis in *Aspergillus flavus*: RT-PCR characterization. J Appl Microbiol 94:953–961

Desjardins AE, Proctor RH (2007) Molecular biology of Fusarium mycotoxins. Int J Food Microbiol 119:47–50

Dobson TA, Desaty D, Brewer D, Vining LC (1967) Biosynthesis of fusaric acid in cultures of *Fusarium oxysporum*. Can J Biochem 45:809–823

Fox EM, Howlett BJ (2008) Secondary metabolism: regulation and role in fungal biology. Curr Opin Microbiol 11:481–487

Haferburg G, Groth I, Mollmann U, Kothe E, Sattler I (2009) Arousing sleep genes: shifts in secondary metabolism of metal tolerant actinobacteria under conditions of heavy metal stress. Biometals 22:225–234

Hill RD, Unrau AM, Canvin DT (1966) The biosynthesis of fusaric acid from $^{14}C$-labeled acetate in *Gibberella fujikuroi*. Can J Chem 44:2077–2082

Ignazio C, Linda MK (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556

Kalyanasundaram R, Sarasvati-Devi L (1955) Zinc in the metabolism of *Fusarium vasinfectum* Atk. Nature 175: 945–947

Mateles RI, Adye JC (1965) Production of aflatoxins in submerged culture. Appl Microbiol 13:208–211

Nirenberg HI, O'Donnell K (1998) New *Fusarium* species and combinations within the *Gibberella fujikuroi* species. Mycologia 90:434–458

Rutherford JC, Bird AJ (2004) Metal-responsive transcription factors that regulate iron, zinc and copper homeostasis in eukaryotic cells. Eukaryot Cell 3:1–13

Scherlach K, Hertweck C (2009) Triggering cryptic natural product biosynthesis in microorganisms. Org Biomol Chem 7:1753–1760

Shwab EK, Keller NP (2008) Regulation of secondary metabolite production in filamentous ascomycetes. Mycol Res 112:225–230

Stipanovic R, Wheeler M, Liu JG, Puckhaber L, Bell A (2008) Biosynthesis of fusaric acid by *Fusarium oxysporum* f. sp. vasinfectum. In: Proceedings of Beltwide cotton conferences

Taboski MAS, Rand TG, Piorko A (2005) Lead and cadmium uptake in the marine fungi *Corollospora lacera* and *Monodictys pelagica*. FEMS Microbiol Ecol 53:445–453

Tan N, Pan JH, Peng GT, Mou CB, Tao YW, She ZG, Yang ZL, Zhou SN, Lin YC (2008) A copper coordination compound produced by a marine fungus *Fusarium* sp. ZZF51 with biosorption of Cu(II) ions. Chin J Chem 26:516–521

Vasavada AB, Hsieh DPH (1988) Effects of metals on 3-acetyldeoxynivalenol production by *Fusarium graminearum* R2118 in submerged cultures. Appl Environ Microbiol 54:1063–1065

Zhang Y, Yang C, Xu F, Liu L, Lin YC (2009) Secondary metabolites of mangrove endophytic fungus B2 in the South China Sea. Acta Sci Nat Univ Sunyatseni (Zhong Da Xue Bao) 48:136–138