Plipastatin A1 produced by a marine sediment-derived *Bacillus amyloliquefaciens* SH-B74 contributes to the control of gray mold disease in tomato

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Abstract

Certain *Bacillus* species have the capacity to produce cyclic lipopeptides and these lipopeptides are promising determinants contributing to the biocontrol of plant diseases. In the current study, a cyclic lipopeptide plipastatin A1 was isolated from the fermentation broth of a marine sediment-derived *Bacillus amyloliquefaciens* SH-B74 by the combination of solid-phase extraction and reversed-phase high-performance liquid chromatography, and its structure was identified by tandem mass spectrometry, high-resolution electro-spray ionization mass spectrometry, and gas chromatography–mass spectrometry together with nuclear magnetic resonance analysis. Moreover, data from activity evaluation revealed that plipastatin A1 has excellent in vitro activity on the suppression of the conidia germination of *B. cinerea*, the causal agent of gray mold disease in tomato. Furthermore, plipastatin A1 can successfully decrease the incidence of gray mold disease on tomato leaves at 50 µM concentration. This study indicates that *B. amyloliquefaciens* SH-B74 appears to be a potentially sustainable pesticide to control gray mold disease in tomato plants, and its cyclic lipopeptide plipastatin A1 plays an important role in the in vitro and in planta biocontrol of *B. cinerea*.

Keywords *Bacillus amyloliquefaciens* · Plipastatin A1 · Mass spectrometry · *Botrytis cinerea* · *Solanum lycopersicum*

Introduction

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*), the causal agent of gray mold disease, is an ascomycete with a necrotic life style (Williamson et al. 2007). It can infect over a hundred host plants and cause severe disease symptoms on them. Germination and adhesion of the conidia of *B. cinerea* have launched the initial infection process, and the further infection followed by penetration of conidia into the host plant surface, invasive growth in the host tissues, and finally sporulation (van Kan 2006; Choquer et al. 2007). Gray mold disease is destructive to vegetables, fruits, and crops, and the pathogen affects greatly on the quality of these products

during the storage processes as well, in terms of *B. cinerea* can grow at a wide temperature range (Droby and Lichter 2004; Asselbergh et al. 2007).

Integrated management of plant diseases has launched great attention as a research focus attributing to the severe side effects triggered by chemical treatment (Weisenburger 1993; Stehle and Schulz 2015; Di Renzo et al. 2015; Nicolopoulou-Stamati et al. 2016). Integrated management of *B. cinerea* needs the combination of chemical pesticides together with biopesticides, which is an alternative control strategy for gray mold disease in agricultural practices. There is several decades time long to develop plant beneficial microbes as control agents against plant diseases (Handelsman and Stabb 1996; Weller 2007). Plant beneficial microbes can produce diverse biocontrol-related secondary metabolites, including special type metabolites dubbed lipopeptides (Raaijmakers et al. 2006; Chen et al. 2007; Loper et al. 2012; Raaijmakers and Mazzola 2012). Lipopeptides have the capacity to reduce surface tension on a hydrophobic interface (Fiechter 1992). Importantly, lipopeptides can interact with multiple cellular membranes of diverse microbes, such as fungi, bacteria, viruses, and plants, due to the hydrophobic and hydrophilic properties of their

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molecular structures (Raaijmakers et al. 2010). Therefore, lipopeptides show antagonistic activities against plant pathogenic microbes and have the ability to trigger plant innate immunity responses, and thus, they are feasible candidates as environmental friendly agrochemicals. More importantly, deciphering the interactions between lipopeptides and plant pathogenic fungi, and developing the biosurfactant-producing bacteria to control plant diseases are important for sustainable agriculture.

Bacillus species are most known microbes used for biocontrol research and agricultural practices, and certain *Bacillus* sp. are potential lipopeptides producers (Ongena and Jacques 2008; Velivelli et al. 2014; Borriss 2011, 2015). The major lipopeptides produced by *Bacillus* sp. are iturins, fengycins, surfactins, kurstakins, and locillomycins (Ongena and Jacques 2008; Hathout et al. 2000; Luo et al. 2015). The chemical structure of a cyclic lipopeptide composes of a peptidic backbone and a fatty acid tail, and the amino acid sequence is generally cyclized. It globally contains *normal*-type, *iso*-type, or *anteiso*-type terminal branches in the fatty acid tail of a lipopeptide. Iturins, especially fengycins, show strong antagonistic activities against filamentous phytopathogens, while surfactins commonly display antibacterial activities, and most importantly, surfactins are elicitors to mount innate immunity in plants and thus decrease the outcome of the invasive growth caused by the infection of pathogenic fungi (Ongena and Jacques 2008).

A *Bacillus* isolate SH-B74 has been previously isolated from a marine sediment sample collected from the South China Sea. In a further study, the strain SH-B74 was identified as *B. amyloliquefaciens* by its morphological, physiological, and biochemical characteristics, as well as 16S rDNA sequencing based phylogeny analysis (GenBank accession number: KC517141) (Niu et al. 2011). In addition, the strain SH-B74 is a potent biosurfactant producer. In the previous work, bacillopeptin A, bacillopeptin B, and a new lipopeptide bacillopeptin B₁ have been purified and characterized from the fermentation broth of the strain SH-B74 (Niu et al. 2011; Ma et al. 2014a). Moreover, the strain SH-B74 can produce the other type lipopeptides (Ma Z. et al., unpublished data), and the structures and bioactivities of these unknown lipopeptides have remained unclear. In the present work, we are interested in the chemical structure and potential biocontrol activity of the uncharacterized lipopeptide from the strain SH-B74, and the detailed activity of the lipopeptide to control *B. cinerea* will be evaluated both in vitro and in tomato plants.

Materials and methods

Microorganisms, cultural media, and growth conditions

B. amyloliquefaciens SH-B74 was routinely maintained on Luria–Bertani (LB) medium (Sambrook et al. 1989). The strain SH-B74 has been deposited in China Center for Type Culture Collection (CCTCC) under No. CCTCC AB 2018012. The component of mineral salts medium (MSM) used in this study was shown previously (Ma et al. 2012). *B. cinerea* (Lab stock) was cultured on potato dextrose agar (PDA), and the fungus was incubated 14 days for sporulation. Unless stated otherwise, microbes used in this study were incubated at 28 °C.

Isolation and purification of lipopeptides

B. amyloliquefaciens SH-B74 was activated on LB plates for 2 days, and a single colony of the strain was inoculated in a 250-mL flask containing 50-mL MSM as a seed culture. The seed culture was incubated on a shaker (180 rpm, 28 °C) for 20 h and then transferred into a 3-L flask containing 1-L MSM and the flasks were incubated on a shaker (28 °C, 180 rpm) for 48 h. Then, the bacterial cultures were harvested after 48-h incubation, and the supernatant was collected after centrifugation at 4000 rpm for 20 min. pH of the bacterial supernatant was adjusted approximately to 2.0 with 6-N hydrochloric acid, and then, the bacterial supernatant was kept overnight at 4 °C. The precipitates from the bacterial supernatant were collected by centrifugation (4000 rpm, 20 min) and were extracted with methanol. The methanol layer from the mixture was collected by centrifugation (4000 rpm, 20 min) and was dried under vacuum; finally, the crude extract of lipopeptides was yielded. The crude extract of lipopeptides was further separated on an SPE flash column (C18, 4 g, 20–45 µm, 100 Å, Agela technologies, Tianjin, China). Briefly, the crude extract of lipopeptides was dissolved in 70% (v/v) methanol/H₂O, and then, the solution was loaded over a SPE flash column under vacuum, and the fractions were obtained by eluting with 80, 90, and 100% (v/v) methanol/H₂O systems, respectively. The fractions from SPE column were collected, dried, and submitted for further bioactivity test and RP-HPLC separation, respectively. A semi-preparative RP-HPLC system (Dionex U3000, Sunnyvale, CA, USA) equipping with a C18 YMC-Pack ODS-A column (5 µm, ϕ 4.6 mm × 250 mm) was used for further analyzing of samples, and a C18 YMC-Pack ODS-A column (5 µm, ϕ 10 mm × 250 mm) was used for further purifying of the interested SPE fraction, respectively.

Mass spectrometry analysis

High-resolution electro-spray ionization mass spectrometry (HRESI MS) experiment of the purified compound was performed on a Bruker micro Q-TOF MS (Germany), and the tandem mass (MS/MS) spectrometric measurement was conducted on an LTQ XL™ linear ion trap mass spectrometer (Thermo Fisher Scientific Inc., USA). The GC–MS analysis was performed on a TRACE GC 2000-TRACE MS system (Thermo Finnigan, USA) equipped with an Agilent J&W HP-5MS column (30 m × 0.250 mm × 0.25 µm, Agilent technologies, Inc., USA).

NMR analysis

The purified compound was dissolved in 0.5 mL CD₃OD-*d*₄ ($\delta_{\text{H}} = 3.30$ ppm, $\delta_{\text{C}} = 49.0$ ppm) and the NMR spectroscopic spectra (¹H-NMR and ¹³C-NMR) of the compound were recorded on a Bruker AV600 spectrometer (Leipzig, Germany, 600 MHz for ¹H-NMR, and 150 MHz for ¹³C NMR, respectively).

Preparation of the TMS derivative

The TMS derivative of fatty acid residue from the purified compound was prepared as shown previously (Ma et al. 2014b). More specifically, RP-HPLC purified compound (1 mg) was decomposed by 6-N hydrochloric acid (1 mL), and this reaction was conducted at 110 °C for 24 h in a sealed glass tube. The mixture was dried under vacuum. 90-µL volume of N, O-Bis (Trimethylsilyl) trifluoroacetamide (TMS, J&K Scientific Ltd., China) and 10-µL volume of anhydrous pyridine were added to the dried mixture, and then, the mixed solution was maintained at the temperature of 70 °C for 3 h to prepare the TMS derivative. Then, the derivative of fatty acid was completely dried under vacuum. Finally, the mixture was dissolved with 100-µL volume n-hexane and centrifuged at 12000 rpm for 5 min; the supernatant was then collected and analyzed by GC–MS.

In vitro biocontrol assay

Stock solutions of the crude extract or purified compound from the strain SH-B74 were prepared in DMSO, and then diluted to the desired concentrations for further bioassays. Unless stated otherwise, 1-, 10-, and 50-µM concentrations of the purified compound were used for bioassays in this study, and the same amount of DMSO was added to corresponding control treatment. In vitro assay of the purified compound was evaluated by assessment of the conidia germination of *B. cinerea*. Spores of *B. cinerea* were collected from PDA plate, and the concentration of conidia was adjusted to 1×10^5 CFU/mL. Conidia suspension was treated

with different concentrations of the purified compound, and 10 µL of the treated spore suspension was dropped on the surface of a microscopic slide and incubated for 12 and 24 h, respectively. Then, the conidia germination was observed by an Olympus BX41TF Microscope (Olympus Corporation, Tokyo, Japan) equipping with an Olympus CAMEDIA C-7070 Wide Zoom Digital Camera. The germination rate was calculated by counting at least 50 spores for each treatment, and the germination rate was calculated from triplicate repeats and was presented as mean ± standard deviation (SD).

In planta biocontrol assay

Tomato line (*Solanum lycopersicum* L. cv. Zhongshu No. 4) was used in biocontrol assay (Gao et al. 2009). The tomato seeds were surface sterilized using 1% (w/v) sodium hypochlorite solution for 2 min, and then, the seeds were rinsed with sterilized water for five times to remove extra sodium hypochlorite on the seed surface. Sterilized seeds were then planted in peat soil and were maintained in a glass house. Plants were watered every 3 days. 5-week-old tomato plants were used for biocontrol assay. The method used for planta biocontrol assay was slightly modified according to a protocol published previously (Asselbergh et al. 2007). More specifically, the third youngest leaves of tomato plants were detached and the leaf discs were prepared by punching out of the detached leaves with a 15-mm diameter plastic pipe. Then, the leaf discs were floated on the sterilized water in round Petri dishes (diameter = 9 cm) and kept on the laboratory bench. A 10-µL droplet of the spore suspension was used for inoculation on the leaf discs of tomato leaves, and the inoculated leaf discs were incubated in the dark. Disease score was evaluated 60-h post-incubation by calculating the percentage of the spreading lesions over the total inoculated leaf discs. The data were collected from triplicate experiments and were presented as means (± SD).

Results

Isolation and purification of compound 1

The crude lipopeptides were obtained from the methanol layer of the precipitates collecting from the bacterial supernatant of *B. amyloliquefaciens* SH-B74 by acid treatment. Then, the crude lipopeptides from the fermentation broth of the strain SH-B74 were dissolved in 70% (v/v) methanol/H₂O and the solution was loaded over an SPE flash column. Subsequently, we have fractionated the crude lipopeptides from the strain SH-B74 to 80, 90, and 100% SPE fractions on the SPE flash column by elution of 80, 90, and 100% methanol/H₂O (v/v), respectively (Fig. S1).

The crude lipopeptides extracted from the bacterial supernatant of *B. amyloliquefaciens* SH-B74 and the SPE fractions of the crude lipopeptides from the strain were tested for inhibiting the conidia germination of *B. cinerea*, respectively. The final concentration of each sample in conidia solution was adjusted to 50 µg/mL. The germination rate of the conidia suspension was then calculated after incubation for 24 h. In addition, the result showed that the crude lipopeptides have significant capacity to inhibit the conidia germination (Fig. S2). Moreover, the inhibition rate of the tested SPE fractions from the crude lipopeptides of the strain SH-B74 displayed that the treatments of the 80 and 90% SPE fractions of the crude lipopeptides have significant activity on the conidia germination of *B. cinerea* (Fig. S2). However, the treatment of the 90% SPE fraction (germination rate = 15.6% ± 4.0) showed a better effect on the inhibition of the conidia germination of *B. cinerea*, compared to the treatment of the 80% SPE fraction (germination rate = 52.3% ± 8.2) separated from the crude lipopeptides of SH-B74.

Therefore, we further purified the main compound (compound **1**) of the 90% SPE fraction of the crude lipopeptide

produced by the strain SH-B74. Compound **1** was afforded on a RP-HPLC system equipped with a C18 YMC-Pack ODS-A column (5 µm, ϕ 10 mm × 250 mm) by eluting via 50% (v/v) acetonitrile/H₂O (0.05% trifluoroacetic acid, TFA, v/v), at a flow rate of 2.5 mL/min, with ultraviolet detection at 220 nm, respectively. The purification of compound **1** was conducted by repeated injections of 90% SPE fraction of the crude lipopeptides produced by strain SH-B74 on the RP-HPLC system, until we have enough quantities of compound **1** for further analysis and assays (Fig. 1).

Mass spectrometry and NMR characterization of compound **1**

The HRESI MS spectrum (Fig. S3) of compound **1** (Fig. 2) showed m/z 1463.8014 ($[M+H]^+$) for molecular formula C₇₂H₁₁₁N₁₂O₂₀ (calculated for m/z 1463.8038). The result was further confirmed by the full scanned mass spectrum of compound **1** (Fig. S4), which showed m/z 1463.75 ($[M+H]^+$) and m/z 1485.58 ($[M+Na]^+$), as well as m/z 732.50 ($[M+2H]^{2+}$), respectively. The ion at m/z 732.50 was chosen as the mother ion for further tandem mass spectrometry

Fig. 1 RP-HPLC profiles of the 90% SPE fraction of the crude lipopeptides obtained from the fermentation broth of *B. amyloliquefaciens* SH-B74 (**a**) and compound **1** isolated and purified from the 90% SPE fraction of the crude lipopeptides of the strain (**b**). The RP-HPLC profiles of the 90% SPE fraction and compound **1** were conducted on a semi-preparative RP-HPLC system (Dionex U3000, Sunnyvale, CA, USA) equipped with a C18 YMC-Pack ODS-A column (5 µm, ϕ 4.6 × 250 mm), by eluting with 55% acetonitrile/H₂O (v/v) and with a flow rate of 0.75 mL min⁻¹

Fig. 2 Chemical structure of plipastatin A1 (**1**) isolated and purified from the fermentation broth of *B. amyloliquefaciens* SH-B74

analysis, which produced a series of product ion fragments. These ion peaks of MS/MS spectrum (Fig. 3a) provided detailed information for elucidating the peptide sequence of compound 1.

The cleavage point of N-terminus of threonine produced a series of *b*-type ion peaks of m/z 1080.42 (b_9), 966.17 (b_8), 803.33 (b_7), 690.25 (b_6), 527.00 (b_5), 398.25 (b_4), 301.75 (b_3), and 230.83 (b_2), respectively, and *y*-type ion peaks of m/z 384.17 (y_1), 498.08 (y_2), 661.33 (y_3), 774.25 (y_4), 938.42 (y_5), 1064.90 (y_6), 1162.67 (y_7), and 1233.58 (y_8), respectively. These ion peaks showed the peptide sequence of Glu₁, Orn₂, Tyr₃, Ile₄, Tyr₅, Gln₆, Pro₇, Ala₈, Glu₉, and

Thr₁₀ (Fig. 3b). Furthermore, the cleavage point of C-terminus of isoleucine produced ion fragments of m/z 1080.42 (b_9), 966.17 (b_8), 803.33 (b_7), 573.08 (b_5), and 502.17 (b_4), respectively, and m/z 384.17 (y_1), 498.08 (y_2), 661.33 (y_3), 891.17 (y_5), 962.58 (y_6), 1188.50 (y_8), and 1350.50 (y_9), respectively. In addition, these ion data confirmed the peptide sequence of Glu₁, Orn₂, Tyr₃, Thr₄, Glu₅, Ala₆, Pro₇, Gln₈, Tyr₉, and Ile₁₀ (Fig. 3c). The N-terminus of proline showed the ions of m/z 225.92, 389.20, 502.17, 1162.67, 1263.42, and 1238.58, 1075.17, 962.58, 301.75, respectively. These ion data confirmed the sequence of Pro₁, Gln₂, Tyr₃, Ile₄, (Tyr-Orn-Glu-A fatty acid)₅, Thr₆, Glu₇, Ala₈ (Fig. 3d).

Fig. 3 MS/MS spectrum (a) and fragmentation ion assignment (b, c, d) of plipastatin A1 (1) resulting from the mother ion m/z 732.65 ($[M+2H]^{2+}$) by tandem mass spectrometry analysis. The MS/MS data indicated different cleavage points existing during the process of tan-

dem mass spectrometric analysis, namely, the N-terminus of threonine (b), the C-terminus of isoleucine (c), and the N-terminus of proline (d), respectively

GC–MS analysis (Fig. 4a) of TMS derivative of the fatty acid residue of compound **1** showed that the major product ion peaks at m/z 401.21 (for $[M-CH_3]^+$), m/z 285.20 (for $[M-CH_2COOSi(CH_3)_3]^+$), m/z 73 (for $[Si(CH_3)_3]^+$), m/z 147 (for $[(CH_3)_2Si^+OSi(CH_3)_3]$), and m/z 233 (for $[(CH_3)_3SiOC^+HCH_2COOSi(CH_3)_3]$), especially m/z 147 and m/z 233, two of which are very characteristic of the TMS derivative of β -hydroxy fatty acid chain (Fig. 4b). The 1H -NMR (Fig. S5) and ^{13}C -NMR (Fig. S6) spectra of compound **1** showed characteristic signals for peptidic compound, such as δ_H from 3.5 to 4.8 ppm, δ_C from 45 to 70 ppm, and 170 to 180 ppm, indicating the peptide bond $-CO-NH-$ probably existed in the compound. These data from NMR measurements show agreement with the results from MS spectra that there is a peptidic sequence in compound **1**. Moreover, the high region of the ^{13}C -NMR spectrum (Fig. S6) of compound **1** showed the information for the terminal branch of the fatty acid residue; more specifically, δ (ppm) 14.5 showed that a *normal*-type methyl carbon exists in the fatty acid residue of compound **1**. Collectively, the fatty acid residue of compound **1** is identified as a saturated *normal*-type C16 β -hydroxy fatty acid (Fig. 4b).

According to the data obtained above, compound **1** composes of Glu₁, Orn₂, Tyr₃, Thr₄, Glu₅, Ala₆, Pro₇, Gln₈, Tyr₉, and Ile₁₀ as the peptide sequence in which Tyr and Ile are cyclized, and a normal-type fatty acid tail (C16:0). Thus, the compound was identified as a lipopeptide belonging to the plipastatin A family, named as plipastatin A1 (Fig. 2). The NMR data of compound **1** were tentatively assigned in Table S1.

Biocontrol assay of plipastatin A1

In further experiments, we wanted to know whether plipastatin A1 has biocontrol capacity to the *B. cinerea* under both in vitro and in plants. First, the in vitro effects of plipastatin A1 on the conidia germination of *B. cinerea* were evaluated by applying together the conidia suspension of *B. cinerea* and various concentrations of plipastatin A1 on the glass slides. The results indicated that plipastatin A1 (**1**) significantly inhibits the conidia germination of *B. cinerea* under in vitro condition; for instance, it shows a significant activity when the concentration of plipastatin A1 is larger than 10 μ M at 12-h post-treatment (Fig. 5a) and 24-h (Fig. 5b) post-treatment, respectively, even 1- μ M treatment of the

Fig. 4 GC–MS analysis of the TMS derivative of C16 β -hydroxy fatty acid residue of plipastatin A1 (**1**) (a), and the product ions of the TMS derivative of C16 β -hydroxy fatty acid generated from GC-MS (b)

Fig. 5 In vitro germination assay of conidia collected from PDA plates of *B. cinerea*. DMSO was used as a control for the assays. Different concentrations of plipastatin A1 were applied to the spore suspension of *B. cinerea*. The germination rate of conidia of *B. cinerea*

was calculated after incubation of the treated conidia for 12 h (a) and 24 h (b), respectively. Different letters show significant differences among different treatments by Turkey's test ($p < 0.05$)

compound is significantly effective to the conidia germination of *B. cinerea* compared with control at 12-h post-treatment (Fig. 5a). In further bioassays, we have applied different concentrations of plipastatin A1 together with the conidia suspension of *B. cinerea* on the leaf discs of tomato plants, to see the planta biocontrol effect of the compound. The results revealed that only the treatment of 50-µM plipastatin A1 could significantly reduce the percentage of the spreading lesions over total lesions compared to the control treatment, while other treatments were ineffective (Fig. 6).

Discussion

In this study, we have isolated and purified a lipopeptide antibiotic plipastatin A1 from the fermentation broth of *B. amyloliquefaciens* SH-B74. To track the lipopeptide produced by the strain SH-B74 which inhibits the conidia germination of *B. cinerea* in vitro, a conidia germination assay was used as a bioactivity-guided approach in this study. We have successfully separated the main compound in a fraction of the crude lipopeptides produced by the strain SH-B74 using an SPE flash column combined with a semi-preparative RP-HPLC system. In addition, the in vitro and in planta biocontrol activities of the purified compound were further evaluated.

Obtaining the crude extract of lipopeptides from *Bacillus* strain is the first step to study these molecules. There have been reported several ways to obtain the crude extract of lipopeptides from the fermentation broth of *Bacillus* lipopeptide producers, such as liquid–liquid extraction, gel filtration chromatography, and size-exclusion chromatography (Kim et al. 2010; Cui et al. 2012; Alajlani et al. 2016).

Fig. 6 Biocontrol capacity of plipastatin A1 against *B. cinerea* in tomato plants (a). Pictures illustrating spreading lesions (b) and non-spreading lesions (c) are shown, respectively. The diameter of the leaf discs showing in Fig. 6b and c is 15 mm. Disease score was evaluated at 60-h post-inoculation (hpi). Each biological repeat contained at least 12 leaf discs from 12 tomato plants. Data were collected from three biological replicates, and asterisk (*) indicates significant difference of the treatment compared with control by Dunnett's test ($p < 0.05$)

However, we have shown that methanol extraction from the precipitates of the *Bacillus* supernatant by the acid treatment is an efficient way to get the crude extract of lipopeptides in this study. A vacuum flash chromatography (VFC) combining with an RP-HPLC system has previously been used to purify the lipopeptides from *Bacillus* sp. (Ma et al. 2012). This study, alternatively, has modified the workflow of the purification of lipopeptides from the strain SH-B74. Namely, we have combined an SPE C18 cartridge with an RP-HPLC system to purify the lipopeptides.

Plipastatins were characterized as a new lipopeptide family from a *Bacillus cereus* strain in the year 1986 (Umezawa et al. 1986). Interestingly, fengycins were reported as a new family of *Bacillus* lipopeptides in the same year (Vanittanakom et al. 1986). Intriguingly, plipastatin A1 and fengycin IX belong to plipastatin and fengycin family, respectively, and the two compounds share the same 2D structure. However, there have the controversial opinions on the structures of plipastatin A1 and fengycin IX for several decades, due to the differences of the stereochemistry of tyrosine (2×) in the peptidic sequences of the two compounds (Umezawa et al. 1986; Vanittanakom et al. 1986). However, an investigation recently demonstrated that plipastatin A1 and fengycin IX are identical compounds, and the stereochemistry of tyrosine in the two compounds prefers to the 3D structure of plipastatin A1 (Honma et al. 2012). We have determined the chemical structure of compound **1** by multiple spectrometric and spectroscopic data. More specifically, the MS/MS and HRESI MS have been used for identifying the amino acid sequence and the molecular formula of compound **1**, respectively. GC-MS and NMR were used for determining the fatty acid part of the compound, respectively. Taken all results together, compound **1** has the same 2D structure as plipastatin A1 and fengycin IX. Although we do not have any data to support the stereochemistry of the amino acids in compound **1**; in this study, it was reasonable and rational that we named compound **1** as plipastatin A1 attributing to the evidence from a report of the Japanese lab (Honma et al. 2012).

Our data showed that the in vitro and in planta activities of plipastatin A1 on the control of gray mold disease are divergent. More specifically, plipastatin A1 shows a significant inhibition activity on the conidia germination of *B. cinerea* under in vitro condition at the concentrations higher than 1 µM at 12-h post-treatment, and 10 µM at 24-h post-treatment compared with control treatments, respectively. However, the concentration of plipastatin A1 needed for successfully controlling gray mold disease on tomato leaves is 50 µM, which was much higher than the effective concentration under in vitro condition, for instance, 1 µM or 10 µM (Figs. 5a, b and 6). This phenomenon possibly shows that the planta interaction of plipastatin A1-tomato-*B. cinerea* is complicated than plipastatin A1-*B. cinerea* under

in vitro condition. It is interesting to decipher the reason that the planta test needs a higher concentration of plipastatin A1 than in vitro to successfully control gray mold disease in tomato. Likewise, we have shown previously that orfamide-type lipopeptides need lower concentration (10 µM) to inhibit appressoria formation of *Magnaporthe oryzae* in vitro, while they need a higher concentration (50 µM) to successfully control rice blast disease caused by *M. oryzae* on rice leaves (Ma et al. 2016).

This study investigated the biocontrol activity of plipastatin A1 produced by the strain SH-B74; however, liquid chromatography-mass spectrometry (LC-MS) data of the crude lipopeptides from the strain SH-B74 show that the strain can possibly produce a group of plipastatins (Ma Z. et al., unpublished data). It could be interesting to elucidate the chemical structures of different homologs of plipastatins produced by the strain SH-B74, and to compare the structure-activity relationships of these compounds in a phytopathogen system.

We have tried to elucidate the lipopeptides produced by the strain SH-B74 for controlling the gray mold disease in tomato in this study. We have shown that 80% and 90% SPE fractions of the crude lipopeptides from the bacterial supernatant of the strain SH-B74 have the significant capacity to inhibit the conidia germination of *B. cinerea* compared to control treatment, while treatment of the crude extract (germination rate = $2.1\% \pm 2.0$) of lipopeptides has showed significant bioactivity on inhibiting of the conidia germination of *B. cinerea* compared to treatments of the 80% (germination rate = $52.3\% \pm 8.2$) and 90% (germination rate = $15.6\% \pm 4.0$) SPE fractions at the same concentration (Fig. S2). This phenomenon, to some extent, appears to reveal that the crude lipopeptides from the strain SH-B74 compose of other ingredients contributing to the capacity of inhibiting the conidia germination of *B. cinerea*.

Plant hormones play important roles in tomato-*B. cinerea* interaction. For instance, it has been shown that abscisic acid (ABA) plays a negative role in the innate immunity of tomato against *B. cinerea*, namely, decreasing the ABA content in tomato plants will lead to the increased level of disease resistance to *B. cinerea* (Asselbergh et al. 2007). We have explained that the biocontrol efficacy of plipastatin A1 on *B. cinerea* is due to the direct antagonistic activity of the compound against the conidia of the pathogen. However, fengycin family lipopeptides from *Bacillus* sp. can trigger disease resistance against *B. cinerea* in tomato plants (Ongena et al. 2007), and plipastatin family lipopeptides can mount disease resistance in grapevine plants (Farace et al. 2015). We have applied the conidia suspension together with plipastatin A1 on the tomato leaf discs in this study. It could be possible that plipastatin A1 also triggers disease resistance against *B. cinerea* on tomato leaves, and it might be that plant hormones (i.e., ABA) are also involved in this type

of interaction. Thus, the detailed mechanisms (not only by direct affecting the conidia germination, but also by inducing disease resistance) underpinning the interaction of plipastatin A1 and *B. cinerea* in tomato plants should be studied further.

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Author contributions Z. Ma conceived and designed the study, conducted the experiments and wrote the paper. J. Hu provided the financial support for this study.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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