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ORIGINAL PAPER

An in vitro system to study cyclopeptide heterophyllin B biosynthesis in the medicinal plant *Pseudostellaria heterophylla*

Wenyan Xu · Hongtao Zhu · Ninghua Tan · Jun Tang · Yingjun Zhang · Ron L. Cerny · Liangcheng Du

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Abstract Plant cyclopeptides are a large group of small molecule metabolites found in a wide variety of plants, including traditional Chinese medicinal plants. Many of the cyclopeptides have highly unusual structures and potent biological activities. However, the majority of the cyclopeptides have not been studied for their biosynthetic mechanisms. In this study, we have established a culture system for the biosynthetic study of heterophyllin B (HB), a cyclopeptide produced by the medicinal plant *Pseudostellaria heterophylla*. We first developed a shoot culture of *P. heterophylla* that produced HB consistently under laboratory conditions. Using ^{14}C-labeled proline as tracer, we showed that labeled HB was produced by the cultured shoots, indicating that this system has de novo biosynthetic activity. Next, we chemically synthesized HB's linear peptide precursor (LHB) and the *N*-acetyl cysteamine thioester of LHB (LHB-SNAC). When LHB-SNAC was incubated with total cell free extracts of the cultured shoots, a small amount of cyclized product (HB), in addition to the hydrolyzed product (LHB), was produced. The in vivo and in vitro results demonstrate the presence of an HB biosynthetic system, which provides insight into the molecular mechanism for plant cyclopeptide biosynthesis.

Keywords Heterophyllin B · Plant cyclopeptide · Biosynthesis · *Pseudostellaria heterophylla* · Traditional Chinese medicine

Abbreviations

HB	Heterophyllin B
LHB	Linear heterophyllin B
LHB-SNAC	*N*-Acetyl cysteamine thioester of LHB
SNAC	*N*-Acetyl cysteamine
NRPS	Nonribosomal peptide synthetases
DCC	*N*,*N*′-dicyclohexylcarbodiimide
Boc	Di-tert-butyl dicarbonate
HOBT	1-Hydroxybenzotriazole
THF	Tetrahydrofuran
CFE	Cell free extract
NAA	Naphthalene acetic acid
IBA	Indole butyric acid
BA	6-Benzyladenine
IAA	Indole-3-acetic acid
KT	Kinetin
ABA	Abscisic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
TFA	Trifluoroacetic acid
TLC	Thin lay chromatography
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethanesulfonylfluoride
BSA	Bovine serum albumin

W. Xu · R. L. Cerny · L. Du (✉)
Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588, USA
e-mail: ldu@unlserve.unl.edu

W. Xu · H. Zhu · N. Tan (✉) · J. Tang · Y. Zhang
State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China
e-mail: nhtan@mail.kib.ac.cn

W. Xu
Graduate University of Chinese Academy of Sciences, Beijing 100049, China

Introduction

Plant cyclopeptides are a large group of small molecule natural products, typically containing 4–10 amino acid residues, isolated from a wide variety of plant species throughout the world (Tan and Zhou 2006). These peptides are also found in traditional Chinese medicinal plants and exhibit numerous biological activities, including anticancer, antibacterial, antifungal, anti-malarial, and sedative effects. Based on their chemical structures and distributions, plant cyclopeptides are grouped into eight types (I–VIII) (Tan and Zhou 2006). Type VIII (cyclotides) is a distinct group with a chain of ~30 amino acid residues, which are much longer than the rest of the cyclopeptides. This group is also the best understood in terms of their biosynthetic mechanism (Craik et al. 2007, 1999; Gillon et al. 2008).

Small molecule peptides can be synthesized ribosomally or nonribosomally. Certain antibiotics, such as microcin MccB17 (Li et al. 1996; Milne et al. 1999), and lantibiotics, such as lacticin 481 (Xie et al. 2004; Xie and van der Donk 2004), are synthesized by translation of mRNA on the ribosome. The type VIII plant cyclopeptides are also synthesized using the ribosomal mechanism (Craik et al. 2007, 1999; Gillon et al. 2008). These peptides are derived from gene-encoded precursors (prepeptides) that undergo post-translational modifications. Another large group of small peptides are biosynthesized using the nonribosomal mechanism (Marahiel et al. 1997). These peptides are assembled by specific enzyme complexes called nonribosomal peptide synthetases (NRPS), independent of the ribosome. Typical NRPSs are modular enzymes composed of a series of functional units (domains). One major distinction between ribosomal peptides and nonribosomal peptides is the structural diversity resulting from the amino acid building blocks. Ribosomal peptides use the 20 proteinogenic amino acids as the basic building blocks, whereas nonribosomal peptides are known to use more than 300 building blocks (Marahiel et al. 1997). This latter feature is also found in many of the type I–VII plant cyclopeptides, which contain nonproteinogenic amino acids and proteinogenic amino acids with modifications, including *β*-hydroxylation, acylation, *N*-methylation, and epimerization (D-amino acids).

So far, the Type VIII cyclopeptides are the only group whose biosynthetic mechanism has been established (Craik et al. 2007, 1999; Gillon et al. 2008). The Type I–VII groups contain many structurally interesting and biologically active cyclopeptides. However, their biosynthetic mechanism remains unclear. In this work, we attempt to establish a culture that is biosynthetically stable under laboratory conditions. Heterophyllin B (HB) was isolated from Tai-Zi-Shen (*Pseudostellaria heterophylla*) (Tan et al. 1993; Morita et al. 1994), a Chinese medicinal plant that is used to treat fatigue, loss of appetite, spontaneous perspiration, dehydration and coughing. Peptide cyclization activity had previously been detected from a crude extract of this plant (Jia et al. 2006). This enzymatic activity catalyzed the cyclization of a linear peptide precursor, NH_2-Gly1-Gly2-Leu-Pro-Pro-Pro-Ile-Phe-COOH (LHB), into the cyclopeptide HB in vitro (Fig. 1). To further explore the molecular mechanism for HB biosynthesis, we have established a culture of the plant that consistently produces HB. Using this stable culture of *P. heterophylla*, we have also prepared total cell free extracts and microsomes to study the in vitro biosynthesis of HB.

Materials and methods

Chemicals and synthesis of LHB and LHB-SNAC

Di-tert-butyl dicarbonate, *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-acetylcysteamine, sodium bicarbonate, potassium carbonate, tetrahydrofuran and dichloromethane were purchased from Sigma. 1-Hydroxybenzotriazole (HOBT) was purchased from Chem-Impex International Ltd. Linear-HB (GGLPPPIF) was commercially synthesized using solid phase peptide synthesis by Suzhou Tianma Pharma Group. To synthesize LHB-SNAC, the N-terminus was Boc-protected following Shendage's method (Shendage et al. 2004) with some modifications. Briefly, 6 µmol of LHB was dissolved in 500 µl of 1 M $NaHCO_3$, and 200 µmol di-tert-butyl dicarbonate was dissolved in 80 µl THF. These two solutions were mixed and shaken over night at room temperature and dried in Speed-Vac at 45°C. The C-terminal thioester activation was performed

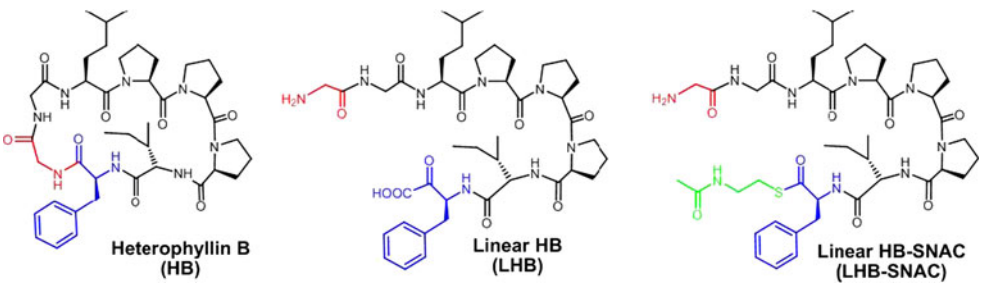

Fig. 1 Chemical structure of heterophyllin B (*HB*), its linear peptide precursor (*LHB*) and the carboxyl-activated peptide precursor (*LHB-SNAC*, thioester of *N*-acetyl cysteamine)

following Wagner's method (Wagner et al. 2006). 12 μmol DCC, 60 μmol HOBT, 60 μmol *N*-acetylcysteamine in 500 μl THF were added into the Boc-protected LHB. The mixture was shaken for 30 min, then potassium carbonate (excess) was added and the mixture was shaken for another 3 h. The mixture was dried in Speed-Vac, and 1 ml 50% TFA in CH_2Cl_2 was added, followed by shaking for 1 h at room temperature. This mixture was dried under flowing air in a fume hood. The final mixture was dissolved in 500 μl extract buffer from the cell free extract (CFE) and stored at −80°C. The identity of the intermediates and product was confirmed by MS analysis.

Cell suspension cultures and shoot cultures of *P. heterophylla*

Roots of *P. heterophylla* were incubated in flasks (250 ml) containing 1/2 MS liquid medium, which were shaken at 180 rpm under lab conditions (22°C, 10 h light and 14 h dark). To induce calluses, the medium was supplemented with naphthalene acetic acid (NAA) 0.5 mg l^{-1}, indole butyric acid (IBA) 0.5 mg l^{-1} and 6-benzyladenine (BA) 0.2 mg l^{-1}. After approximately 30 days, calluses started to appear on the roots. Calluses (0.5 g $flask^{-1}$) were cut and transferred to fresh 1/2 MS liquid medium (30 ml) containing glass beads (3 mm diameter) and shaken at 250 rpm overnight. The cell suspension was generated by continuous shaking of the cultures at 180 rpm for another week. The shoot cultures were prepared from roots cultured in 1/2 MS liquid medium without hormones, which led to the roots budding in about 20 days. The buds were cut and transplanted into MS solid medium containing various combinations of plant hormones, including NAA, indole-3-acetic acid (IAA), IBA, kinetin (KT), BA, abscisic acid (ABA), and 2,4-dichlorophenoxyacetic acid (2,4-D), as shown in Table 1. The cultures were used in the following experiments. Under laboratory conditions, these buds developed into young shoots (2–3 cm long) within 14–15 days and were used in the assays. To regenerate roots, young buds (1 week old, 0.5 g $flask^{-1}$) were cut and transferred to fresh 1/2 MS liquid medium (30 ml) containing NAA 0.5 mg l^{-1}, IBA 0.5 mg l^{-1} and BA 0.2 mg l^{-1}.

Preparation of cyclopeptide extracts

To study the trend of HB content in *P. heterophylla* in different seasons, whole plants were collected from a commercial planting field in Yuqing, Guizhou Province, China, from March 2005 to February 2006. In detail, plants were collected about every 10 days between March 2005 to June 2005, in which plants were found growing above the ground, and about every month during July 2005 to February 2006 in which plants were in dormancy underground. Three replicates were collected and analyzed at each time point. To extract the cyclopeptides, 10 g of dry plant samples or 1 g of cell/shoot culture were extracted three times with ethyl acetate (10 × volume against weight) for 2 h. The extracts were combined, filtered, and concentrated on a Rota-vapor. Each of the dried extracts were washed twice with 20 ml of petroleum ether, and the residues were dissolved with 20 ml of ethyl acetate. The solution was again concentrated on a Rota-vapor, and the extract was redissolved in an appropriate amount of methanol to a final concentration of 5 mg ml^{-1}.

HPLC analysis

HPLC analysis was utilized in the quantification of HB in medicinal material following the methods outlined in (Yang et al. 2009): Cyclopeptides were separated on an HPLC column (Agilent ZORBAX SB-C18X, 150 mm by 4.6 mm, 5 μm). The HPLC system was Hewlet Packard HP Series1100, with a detection wavelength of 210 nm and flow rate of 1 ml min^{-1}. The mobile phases were water and acetonitrile (73:27, v/v). In 2010, a new modified HPLC-analysis of HB was established in the Pharmacopoeia of the People's Republic of China. We chose this method for the analysis of HB in cultured shoots, roots and also for in vivo

Table 1 Combinations of plant growth regulators included in a solidified Murashige and Skoog (MS) used for proliferating shoot cultures of *Pseudostellaria heterophylla*

No.	NAA	IAA	IBA	KT	BA	ABA	2,4-D
ZP01	0	0	0	0	1.5	0	0
ZP02	1.5	0	0	0	0	0	0
ZP03	0.8	0	0.5	0	0	0	0
ZP04	0.8	0	0.5	0	0	0	1.0
ZP05	0.8	0	0.5	0	0	0.3	0
ZP06	0.8	0	0.5	0	0.2	0	0
ZP07	0.6	0.5	0	0	0.1	0	0
ZP08	0.5	0.5	0.5	0.5	0	0	0
ZP09	0.5	0.5	0.5	0.5	0.5	0	0
ZP10	0.5	0.5	0.5	0.5	0.2	0	0

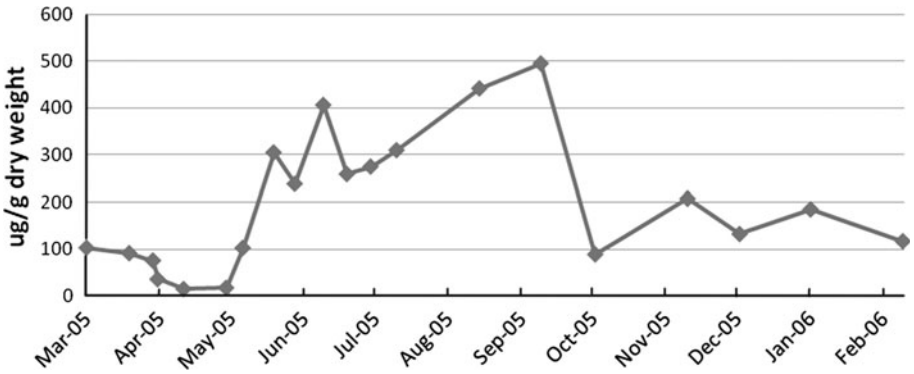

Fig. 2 HB content in *P. heterophylla* whole plant at different growing seasons. Three replicates were analyzed at each time point, and the relative standard deviations were lower than 7%. The content (in μg/g dry material) fluctuated between 14.8 and 494.5 μg/g dry material

and in vitro reactions. The extract was separated on an HPLC column (Alltima C18LL, 250 mm by 4.6 mm, 5 μm) for cyclopeptide analysis. The HPLC system was ProStar model 210 (Varian, Walnut Creek, CA), with a detection wavelength of 210 nm and a flow rate of 1 ml/min. The mobile phases were water-trifluoroacetic acid (TFA; 0.25%, v/v) (mobile phase A) and acetonitrile-TFA (0.25%, v/v) (mobile phase B). The gradient of the solvents (v/v) was as follows: 0–5 min, 5% B; 5–25 min, 5–65% B; 25–35 min, 65% B; 30–33 min, 65–100% B; 33–35 min, 100%; 35–36 min, 5% B. Standard HB, which was purified in Tan group, was available (purity >98% by HPLC) and used for both quantification and as a reference.

De novo biosynthesis of HB in cultured shoots of *P. heterophylla* using ^{14}C-proline as a tracer

Shoots of *P. heterophylla* were cultured in 1/2 MS solid medium at room temperature until they were 2–3 weeks old (2–3 cm long). Each shoot (~20 mg) was cut at the end of the second pair of leaves and transferred into a transparent test tube (sterile polystyrene with cap, 12 × 75 mm) containing 95 μl 1/2 MS liquid medium and 5 μl ^{14}C-Pro (0.1 μCi μl^{-1}). The shoots were incubated at room temperature for 24 h and extracted with 1 ml methanol. The extracts were concentrated and redissolved in 20 μl methanol. A fraction (3.4 μl) of the extracts was loaded to a TLC plate (Silica Gel F, Analtech), which was developed in a solvent composed of chloroform and methanol (9:1, v/v). An extract from similarly treated shoots, but without ^{14}C-Pro feeding, was also loaded on to the same TLC plate and developed alongside the ^{14}C-Pro treated samples. To visualize, the plate was exposed to X-ray film at room temperature for 2 weeks. Upon identification of radioactive bands, the corresponding regions of the non-^{14}C-Pro treated sample were recovered from the TLC plate. The silica powder from each of the collected regions was soaked in methanol, and the extracts were analyzed by HPLC and MS.

Preparation of cell free extracts

To prepare the cell free enzyme extract (CFE), shoot culture (780 mg) from *P. heterophylla* was flash frozen by liquid nitrogen and ground in a pre-chilled mortar with a pestle. The powder was transferred into a container containing 4 ml CFE buffer (25 mM MOP pH 7.0, 1 mM NaCl, 2 mM DTT, 1 mM EDTA, 1 M sucrose, 1 mM PMSF). The mixture was incubated at 4°C for 2 h and centrifuged at 16,100×*g* for 5 min. Then $(NH_4)_2SO_4$ was added into the supernatant until 100% saturation was reached at 4°C. The solution was incubated at 4°C for another 30 min and centrifuged at 16,100×*g* for 5 min. The precipitate was collected and redissolved in 400 μl CFE buffer. This total cell free extract was used for in vitro assays.

Preparation of microsomes

Shoots of *P. heterophylla* (~2 weeks old, 5 g) were flash frozen with liquid nitrogen and ground with polyvinylpolypyrrolidone (0.5 g) in a pre-chilled mortar with a pestle. The powder was transferred into a container and mixed with 10 ml ice-cold microsome buffer (25 mM MOP pH 7.8; 100 mM ascorbic acid; 2 mM DTT; 2 mM EDTA; 250 mM sucrose; 5 mg ml^{-1} BSA; 1 mM PMSF). The mixture was incubated at 4°C for 2 h and filtered through double layer cheese cloth. The filtrate was centrifuged at 10,000×*g* for 15 min at 4°C. The supernatant was collected, and microsomes were prepared from the supernatant by ultracentrifugation at 180,000×*g* for 45 min. The microsomal pellet was resuspended in 60 μl of microsome buffer supplemented with an additional 100 mg ml^{-1} BSA. The microsomes were separated into aliquots and used for in vitro activity assays.

Activity assay in CFE and microsomes

CFE or microsomes (4 mg equivalent of the shoots) were incubated with LHB-SNAC (600 mM) in a reaction mixture (33 μl) at 37°C for 2 h. Denatured CFE or microsomes

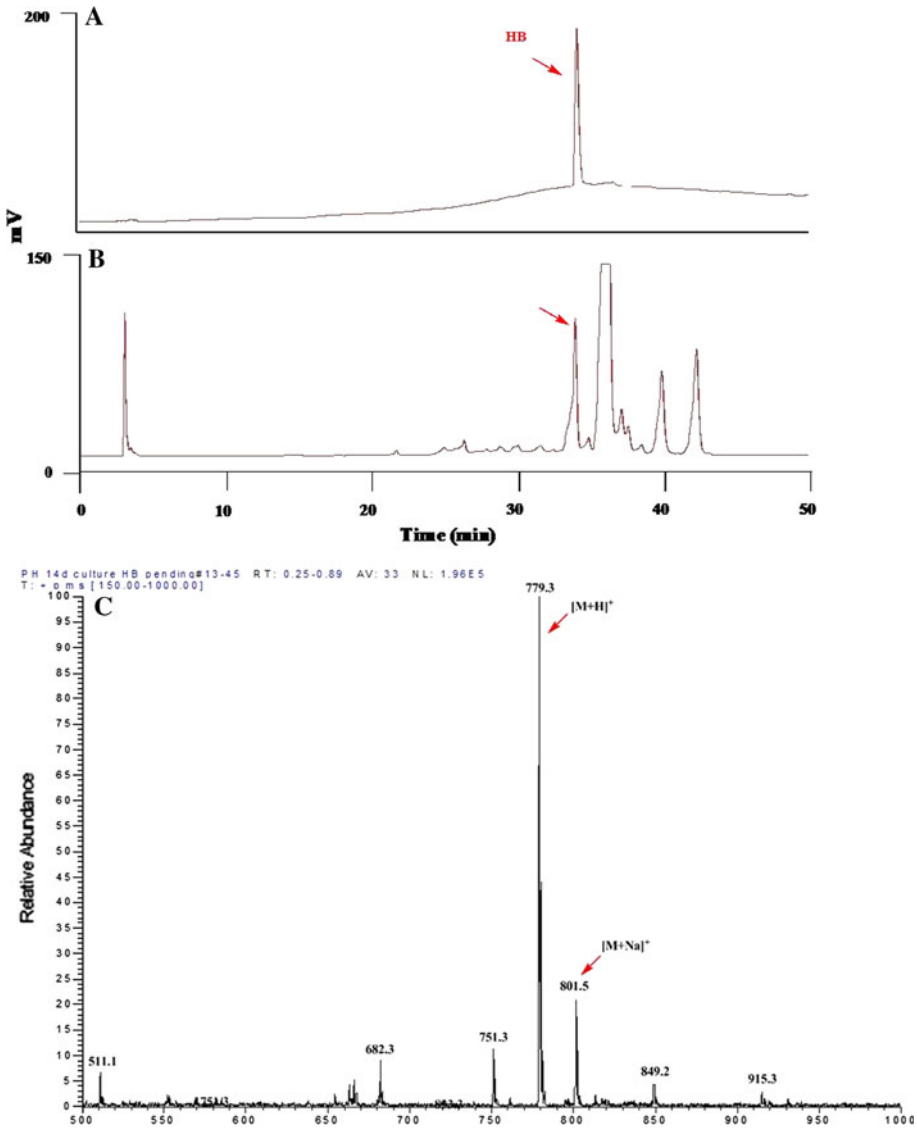

Fig. 3 HPLC and MS analysis of HB production in *P. heterophylla*. **a** Standard HB; **b** extract from shoot culture of *P. heterophylla*; **c** mass spectrum of the peak at 34.0 min in **b**

(boiled 10 min) served as a negative control for the enzyme activity assays. The reaction was quenched by adding 100 µl acetone. The samples were stored at −20°C until analyzed. To detect the cyclized product, 1 µl of the sample was diluted in 100 µl acetonitrile and analyzed by MS.

Results and discussion

We first examined the trend of HB production in *P. heterophylla* during different seasons. As shown in Fig. 2, the HB content fluctuated throughout the seasons, but the general trend was that the plants had a low content (~100 µg per g dry weight) of HB during the winter to early summer season (January to early May) and a high content (300–500 µg per g dry weight) of HB during the summer (late May to early September). There was a period of rapid increase during early May, and HB continued to accumulate in the summer and peaked at mid fall (~500 µg per g dry weight). This suggests that the biosynthetically most active stage for HB in the plant is likely May when the plants are in the post-budding stage. Thus, we collected plants in early May for cell/tissue culture development.

In order to study the HB biosynthetic mechanism, we needed to develop a *P. heterophylla* culture that was consistently able to produce HB under laboratory conditions. We first attempted to develop a cell suspension culture. Roots from the post-budding plants were used as the starting material. Calluses were induced in 1/2 MS liquid medium containing 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} BA, and suspended cells were obtained by shaking the calluses with glass beads. The cell suspension was analyzed for HB production. HPLC results revealed that HB was produced by the cell suspension culture (data

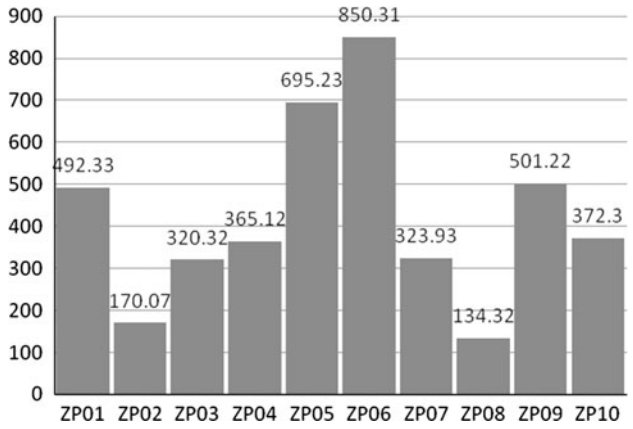

Fig. 4 HB content in shoot culture of *P. heterophylla*. Two replicates of the same culture conditions (ZP01 to ZP10, in Table 1) were analyzed, and the relative standard deviations were lower than 5%. The yield (μg/g dry materials) was the highest in ZP06

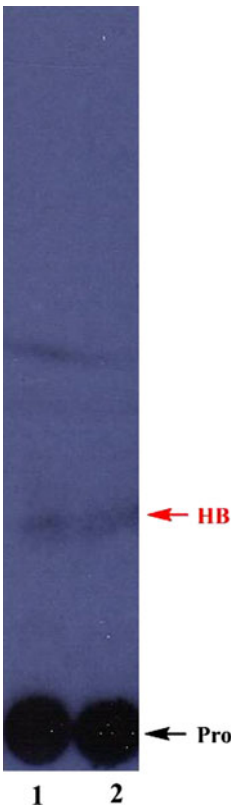

Fig. 5 De novo biosynthesis of HB in shoot culture of *P. heterophylla*. Two separate feedings (*1*, *2*) were conducted and the extracts were analyzed by TLC, and the radioactive signals were visualized by exposure to an X-ray film. The bands corresponding to proline and HB are indicated with an *arrow*

not shown). Under the conditions used, the yield of HB was estimated to be 2.5 μg ml^{-1} per culture for the first generation and 1.5 μg ml^{-1} for the second generation. This demonstrates that the cell suspension culture of *P. heterophylla* is biosynthetically viable. However, the yield of HB decreased steadily in the following generations of cell cultures, indicating that they are not ideal for the biosynthetic study.

In order to develop a biosynthetically reliable system, we next tested a shoot culture that was induced from the roots. We tested 10 different hormone combinations (ZP01-10, Table 1) in MS medium to find out the best culture condition (ZP06) for HB production. HB production in the shoots was analyzed by HPLC (Fig. 3a, b). The ethyl acetate extract from the shoots gave several main peaks on HPLC, one of which was at 34.0 min. This peak shared the same retention time as the standard HB. The mass spectrum of the compound at 34.0 min yielded a base ion at *m/z* 779.3, which is consistent with the [M + H] ion of HB (Fig. 3c). Another major ion at *m/z* 801.5 is consistent with the [M + Na] ion of HB. The yield of HB in shoots varied from 134 μg g^{-1} dry weight (in ZP08) to 850 μg per g dry weight (in ZP06) (Fig. 4). The results indicate that the cultured shoots are prolific producers of HB. The yield is generally comparable to or higher than that in the whole plant, and under certain conditions (ZP06 and ZP05) the yield of HB in shoot cultures is significantly higher than the HB content in the whole plant (Fig. 2). The shoots can be easily maintained in culture flasks under laboratory conditions by transplanting them approximately once a month. HB production in the shoots remained consistent even after more than 20 generations of transplantation. Furthermore, the shoots can regenerate roots by culturing the buds in 1/2 MS liquid medium that contain the appropriate hormones. These results show that the shoot culture has the desired properties to be utilized as a model HB biosynthetic system.

After the shoot culture was established, we tested its de novo biosynthetic activity using ^{14}C-proline as a tracer. The cyclopeptide extracts were loaded onto a TLC plate to separate HB from other metabolites. The TLC plate indicated that the strongest signal remained at the site of sample loading, which was the same as the ^{14}C-proline standard. This indicates that the majority of the radioactivity still remained in proline after the 24-h feeding. However, two relatively weak signals with an R_f of 0.26 and 0.49 appeared on the film (Fig. 5). The band with an R_f of 0.26 and found to be HB, as confirmed by HPLC and MS analysis, was recovered from the corresponding region on the same TLC plate. The results show that proline is a precursor of HB, although the incorporation rate appears to be low. Considering the results of cell suspension cultures, shoot cultures and proline labeling experiment, the biosynthesis of HB may be located in the aerial parts and transform into roots.

With the in vivo activity confirmed in the shoot culture, we next tested whether an in vitro enzymatic system could be established from this culture. We assayed the activity for peptide cyclization in the total cell free extract (CFE) (Jia et al. 2006). The carboxyl group of LHB was first activated as a thioester (LHB-SNAC). MS analysis of the reactions showed two major peaks at *m/z* 898.3 and 920.4,

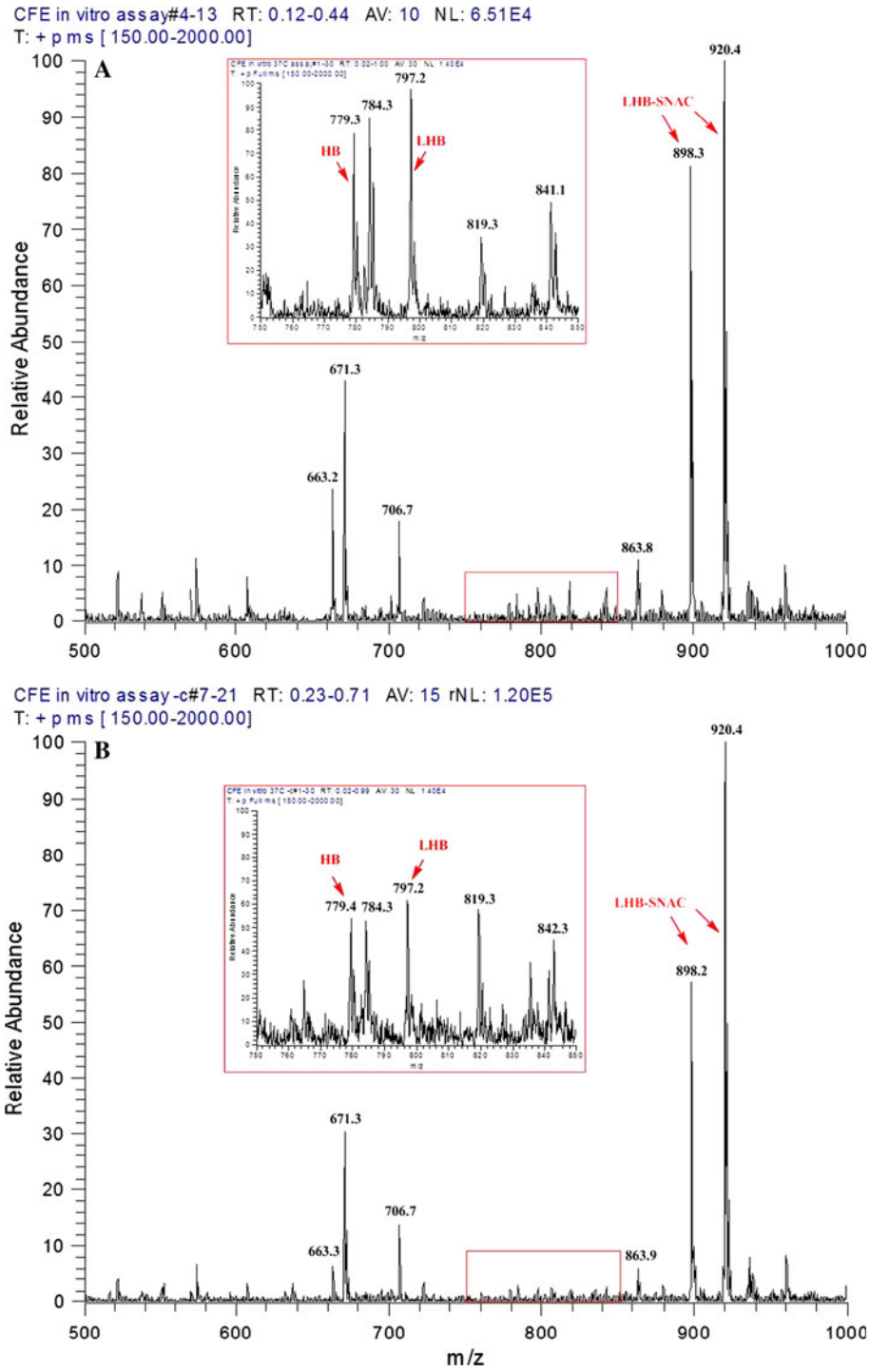

Fig. 6 MS analysis of in vitro reaction products. **a** Reaction with cell free extract; **b** reaction with boiled cell free extract. The *inset* in each of the MS is a zoom-in of the region between *m/z* 750-850 of the full spectra as indicated by the *rectangle*

which correspond to the [M + H] ion and [M + Na] ion, respectively, of the substrate LHB-SNAC (Fig. 6a). The cyclized product appeared as a small peak at *m/z* 779.3, which is the [M + H] ion of HB. In addition, two minor peaks at *m/z* 797.2 and 819.3, which correspond to the [M + H] ion and [M + Na] ion of the hydrolyzed product LHB, were also present. Interestingly, the cyclized product HB and the hydrolyzed product LHB were also detected in the reaction when boiled CFE was used (Fig. 6b). These results suggest that LHB-SNAC had undergone non-enzymatic cyclization and hydrolysis under the reaction conditions. This is not very unusual for the labile thioester LHB-SNAC that tends to undergo non-enzymatic hydrolysis (Ehmann et al. 2000; Hoyer et al. 2007). In addition, it is likely that the CFE contained hydrolytic enzymes that hydrolyzed the thioester bond of LHB-SNAC. The

intensity of the product peaks in the reaction with boiled CFE was about 2/3 that of the peaks in the reaction with native CFE. This suggests that enzymatic activity, albeit weak, was present in this CFE. To further test this cyclization activity, we prepared microsomes from the shoot culture. When LHB-SNAC was incubated with the microsomes, only the hydrolyzed product LHB, but not the cyclized product HB, was detected (data not shown). This suggests that the cyclization activity is most likely located in the soluble fraction, not in the membranes.

Studies of secondary metabolism using in vitro cultures are useful as they allow for controlling enzymes and/or substrates involved in synthesis of metabolites (Sood and Chauhan 2010; Bernabe-antonio et al. 2010; Pati et al. 2011; Nasim et al. 2011). Although many cyclopeptides have been isolated from various plants, the biosynthetic mechanism for the majority of the peptides remains unclear. One of the bottlenecks in studying plant cyclopeptide biosynthesis is the lack of a stable culture/enzymatic system that can be reliably used in in vivo and in vitro studies. Here, we used *P. heterophylla* as a model system to develop a shoot culture that is biosynthetically stable for over 20 generations. This convenient system can be used for feeding experiments as well as biosynthetic enzyme isolation and identification. The establishment of this system will help elucidate the molecular mechanistic aspects in HB peptide chain origin, assembly, and cyclization. Secondary metabolism is regulated by several factors (Albert et al. 2010), including seasonal and tissue-specific expression (Balestrazzi et al. 2011), thus biochemical and molecular studies (Lokhande et al. 2010; Mohanty et al. 2011) will aid in mining additional information.

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