CHAPTER III

MATERIALS AND METHODS

1. Chemicals

All chemicals used were standard commercial products of analytical grade from the companies: Biomol, Boehringer Mannheim, Fluka, Boehringer Ingelheim, Merck, Roth and Sigma. Organic solvents used in this study were all reagent grade or better. Standard and radiolabeled compounds were important and used in this study as follows.

Standard compound:	Plumbagin (Sigma)
	Acetyl-Coenzyme A (CoA) (Sigma)
	Malonyl-CoA (Sigma)
Radiolabeled compound:	[1-^{14}C] Acetyl-CoA (4 mCi/mmol)
	(Biotrend Chemikalien)
	[2-^{14}C] Malonyl-CoA (55 mCi/mmol)
	(Biotrend Chemikalien)

2. Plant tissue culture techniques

2.1 Plant materials

Plumbago indica Linn. was grown in the open field of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok and the greenhouse of the Leibniz Institute of Plant Biochemistry, Halle, Germany, at 24$\pm$2 °C with 18 h of light and 50% humidity.

2.2 Nutrient media

Standard basal media used in this study were Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1965), Gamborg (B5) (Gamborg et al., 1968) and Murashige and Skoog (MS) (Murashige and Skoog, 1962). Plant growth regulators used in this study are listed below. For semi-solid media, 0.6% (w/v) agar was added to the nutrient solutions. The pH of the medium was adjusted to 5.5 for B5 medium, 5.6 for LS medium and 5.8 for MS medium before autoclaving.

Auxin:	Indole-3-acetic acid (IAA)
	α-Naphthaleneacetic acid (NAA)
	2,4-Dichlorophenoxyacetic acid (2,4-D)
Cytokinin:	6-Benzylaminopurine (BA)
	Kinetin-6-furfurylaminopurine (kinetin)

2.3 Culture conditions

The *in vitro* cultures of *P. indica* were maintained in a culture room with a 16 h photoperiod at 24 °C.

2.4 Preparation of explants as starting materials

Young stems (ca 10 cm in length) with leaves removed and young leaves of *P. indica* were dipped into 70% ethanol for 1 min, surface sterilized with 1.0% (v/v) sodium hypochlorite containing a few drops of Tween 20 and shaked gently for 5 min. After immersion for 5 min, young leaves were washed three times in sterile distilled water. A piece of stem was cut into sections of stem (1 cm in length) and nodal segments (1-1.5 cm in length). The stem segments were used as the explants for callus induction. The nodal segments were used as starting explants for

micropropagation. For root induction from leaves, sterilized young leaves were cut into segments (0.5 x 0.5 cm^2) and placed on medium.

2.5 Establishment of callus culture

Young stem segments of *P. indica* were placed on MS medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l BA. Callus induced from stem explants was transferred and subcultured on the same medium every 4 weeks under a 16 h photoperiod at 24 °C.

2.6 Establishment of root culture

The root cultures of *P. indica* were first initiated from young leaf segments by culturing on B5 medium supplemented with 1.0 mg/l NAA and 0.1 mg/l kinetin as previously reported (Panichayupakaranant and Tewtrakul, 2002). The induced roots were excised from leaf segments and cultured in 100 ml Erlenmeyer flasks containing 25 ml of MS liquid medium. The flasks were incubated on a rotary shaker at 80 rpm with 16 h photoperiod at 24 °C. The root cultures of *P. indica* were maintained under these conditions and subcultured every 4 weeks.

2.7 *In vitro* propagation

In vitro propagation of *P. indica* was achieved through tissue culture. Nodal segments used as starting explants were cultured on two different media for shoot induction. The shoot induction media were 1) MS medium supplemented with 0.1 mg/l indole-3-acetic acid (IAA) and 3.0 mg/l BA and 2) MS medium supplemented with 2.0 mg/l BA. The regenerated shoots (1-1.5 cm in length) were transferred onto LS medium for rooting. For long term culture, the nodal segments (1-1.5 cm in length) excised from plantlets were cultured on LS medium and subcultured every 12 weeks.

3. Detection and determination of plumbagin

3.1 Plant material

Plant tissue cultures of *P. indica* (section 2.5-2.7) and roots of *P. indica* were collected for detection of plumbagin. All samples were lyophilized or dried overnight in a hot air oven at 50 °C.

3.2 Determination of plumbagin

3.2.1 Sample preparation

Dried samples of *P. indica* were ground to fine power in a mortar. The ground samples (200 mg each) were extracted three times with 10 ml of 95% ethanol at 50 °C. After passage through a filter paper, the pooled ethanolic fractions were evaporated under vacuum. The residue was dissolved in 1 ml of methanol and plumbagin was analyzed by high performance liquid chromatography (HPLC).

3.2.2 HPLC analysis

Plumbagin was detected by HPLC, in which 25 μl of the methanol solution was injected onto a Lichrospher 60, RP-select B column (250 x 4 mm, 5 μm) (Merck) connected with Hewlett Packard Series 1100 HPLC. The solvent systems were (A): 98% (v/v) H_2O, 2% (v/v) acetonitrile and 0.01% (v/v) acetic acid and (B): 98% (v/v) acetonitrile, 2% (v/v) H_2O and 0.01% (v/v) acetic acid, with the following gradient: 0-25 min 0-60% B, 25-30 min 60% B, 30-32 min 60-100% B, 32-35 min 100% B, 35-37 min 100-0% B and 37-40 min 0% B at a flow rate of 1 ml/min. Plumbagin was monitored at 270 nm using authentic plumbagin as reference standard.

3.3 Quantitative analysis of plumbagin

Lyophilized samples of *P. indica* (section 3.1) were extracted with 100 ml of methanol. The methanolic extracts were evaporated under vacuum and the residues were then dissolved in 5 ml of methanol. Plumbagin was quantified by HPLC using a Symmetry C18 column (Waters). The solvent systems were H_2O containing 0.05% (v/v) trifluoroacetic acid (TFA) (A) and acetonitrile containing 0.05% (v/v) TFA (B) with the following gradient: 30 min 70% B, 35 min 100% B, 40 min 100% B, 41 min 5% B, and 46 min 5% B, flow rate 1 ml/min with detection at 284 nm.

3.4 Identification of plumbagin, plumbagic acid and plumbagic acid glycoside

To identify plumbagin, plumbagic acid and its glucoside, the preparation of crude extract from dried roots of *P. indica* plantlets was scaled-up. Four hundred milligrams of ground root were extracted three times with 20 ml of ethanol at 50 ºC. After evaporation of the crude extract, the residue was further purified by preparative HPLC under the same conditions as described in section 3.2.2. The peaks of interest were collected after elution from the HPLC column at retention time (Rt) =12.878 min for plumbagic acid glycoside, Rt = 17.150 min for plumbagic acid and Rt = 25.251 min for plumbagin. After freeze-drying, the purified compounds were dissolved in methanol. Plumbagin was confirmed by comparison of retention time and GC-MS spectrum with an authentic plumbagin standard. The GC-MS measurements of plumbagin were performed on a Voyager/Trace GC 2000 (Thermo Quest CE Instruments). Plumbagic acid and plumbagic acid glucoside were identified by LC-MS and LC-MS/MS using a Finnigan MAT TSQ 7000 instrument coupled to Micro-Tech Ultra-Plus MicroLC system equipped with RP18-column of the Leibniz Institute of Plant Biochemistry, Halle, Germany.

4. Detection of polyketide synthase activity in crude protein extracts

4.1 Preparation of crude protein extracts

The crude protein extracts were prepared from various tissue cultures of *P. indica*. Before preparation of crude protein extract, *P. indica* root culture was harvested and washed with tap water to remove remaining liquid medium. Fresh tissue was fast frozen by liquid nitrogen and ground to a fine power in a pre-cooled mortar. To 1 g of frozen ground powder of cells 0.1 g quartz sand were added, and the mixture was extracted with extraction buffer (0.1 M potassium phosphate (KPi), pH 7.0) by grinding to fine suspension. The suspension was centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was transferred to a new tube and centrifuged again until it was clear. To remove the phenolic compounds, 0.25 ml of absolute ethanol was added to 2.25 ml of the clear supernatant and mixed by inverting. The mixture was applied to an equilibrated Sephadex G-25 (PD-10 column, Amersham Biosciences) for desalting and eluted with the extraction buffer.

4.2 Detection of polyketide synthase activity

Polyketide syntase (PKS) assay with a crude protein extract contained 200 mM KPi buffer (pH 7.0), 1 mM NADPH, 30 μM acetyl-CoA, 70 μM [2-^{14}C] malonyl-CoA (80,000 dpm) and 50 μl a desalted crude protein extract (section 4.1) in a 100 μl reaction volume. The assay mixture was incubated at 30°C for 2 h or overnight. The reaction was stopped by the addition of 10 μl of 10% (v/v) HCl. The products were extracted twice with 200 μl of ethyl acetate, and evaporated to dryness. The residue dissolved in 10 μl ethyl acetate and separated by thin layer chromatography (TLC). RP18 plates (Merck) were used for reversed-phase TLC with methanol: H_2O: acetic acid (75:25:1) as solvent. For normal phase TLC, silica gel 60

F254 plates (Merck) were developed in ethyl acetate: methanol: H_2O (100:16.5:13.5) or toluene: formic acid (99:1). The radioactive products were visualized by phosphorimaging (Typhoon 9410, Amersham Biosciences).

5. Molecular cloning techniques

5.1 Microorganisms

***E. coli* DH5α** (Clontech)

Genotype: F^- *φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44λ⁻thi-1 gyrA96 relA1*

***E. coli* XL-Blue MRF'** (Stratagene)

Genotype: Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44recA1 gyrA96 relA1 lac* [F'*proAB lac^{q}ZΔM15*] Tn*10* (Tet^r)]

***E.coli* SOLR™** (Stratagene)

Genotype: $e14^-$($McrA^-$)Δ(*mcrCB-hsdSMR-mrr*) 171 *sbcC recB recJ uvrC* : :Tn5(Kanr) *lac gyrA96 relA1 thi-1 endA1* λ^R [F'*proAB lac^{q}ZΔM15*]Su^-(nonsuppressing)

***E. coli* BL21 Star™ (DE3)** (Invitrogen)

Genotype: F^- *ompT hsdS$_B$* ($r_B^- m_B^-$) *gal dcm rne131* (DE3)

5.2 Media

The composition of various media are described in "Molecular Cloning. A Laboratory Manual" (Sambrook et al., 1989) as follows:

Luria-Bertani (LB) broth	1.0 % (w/v) tryptone
	0.5 % (w/v) yeast extract
	1.0 % (w/v) NaCl (adjust pH 7.0)

LB agar	LB broth with 1.5% (w/v) agar
NZY agar	1.0 % (w/v) NZ-amine
	0.5 % (w/v) yeast extract
	0.5 % (w/v) NaCl
	0.2 % (w/v) $MgSO_4.7H_2O$
	1.5 % (w/v) agar
	pH 7.5 (adjusted with 1 M NaOH)
NZY top agar	1.0 % (w/v) NZ-amine
	0.5 % (w/v) yeast extract
	0.5 % (w/v) NaCl
	0.2 % (w/v) $MgSO_4.7H_2O$
	0.7 % (w/v) agarose
	pH 7.5 (adjusted with 1 M NaOH)
SOB medium	2.0 % (w/v) tryptone
	0.5 % (w/v) yeast extract
	0.05 % (w/v) NaCl
	2.5 mM KCl
	Adjust pH 7.0, sterilize by autoclaving and add sterile $MgCl_2$ and $MgSO_4$ (each 10 mM).
SOC medium	SOB medium supplemented with 20 mM glucose (sterile added after autoclaving, together with magnesium salts).

5.3 Enzymes

Taq DNA polymerase	prepared by Dr. J. Ziegler (Leibniz Institute of Plant Biochemistry, Halle/Saale, Germany)
Pfu DNA polymerase	(Promega)
T_4 DNA ligase	(Promega)
M-MLV Reverse Transcriptase	(Life Technologies)
Restriction endonucleases	(Life Technologies and New England Biolabs)

5.4 Molecular biology kits

cDNA synthesis:	Superscript II reverse transcriptase (Life Technologies)
mRNA purification:	Oligotex^{TM} mRNA Midi Kit (Qiagen)
cDNA library:	cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene)
DNA purification:	MiniElute Gel Extraction Kit (Qiagen) QIAprep Spin Miniprep Kit (Qiagen)

5.5 Special chemicals used in the molecular biology procedures

Blue-white selection:	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Biomol)
Protein expression:	Isopropyl-n-D-thiogalactoside (IPTG) (Biomol)

Protein purification:	TALON Metal Affinity Resin (Clontech)
	PD-10 Columns (Amersham Biosciences)
DNA sequencing:	Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems)
Antibiotics:	Ampicillin (Sigma)
	Kanamycin (Sigma)

5.6 Nucleic acids

DNA Length standard:	100 bp DNA-ladder (Life Technologies)
Nucleotides:	deoxynucleotides (dATP, dCTP, dGTP, dTTP) (Life Technologies)
Nucleotide primers:	primers for PCR synthesized at MWG-Biotech AG and Biomers (Germany)

5.7 Plasmids

pGEM-T Easy Vector System (Promega)

This vector was used to subclone the *Taq* DNA-polymerase amplified DNA fragments. This linear 3015 bp vector has a 3' terminal thymidine overhang. *Taq*-polymerase in the PCR reaction unspecifically adds a 3'-deoxyadenosine overhang to the amplified DNA fragments, which enables a straightforward ligation into pGEM-T Easy vector. The selection of transformed bacteria is ensured by the presence of an additional gene in this plasmid that codes for the ampicillin resistance protein.

pBluescript SK(-) (Stratagene)

This 2958 phagemid occurs as the product of *in vivo* excision from a λ-phage. This vector enables a blue-white screening through the complementation of β-galactosidase enzyme and carries a selection marker gene for ampicilin resistance.

pET-14b (Novagen)

pET-14b (4671 bp; selection marker: ampicillin resistance) was used as vector for the expression of recombinant proteins in *E. coli*.

5.8 Equipments

Centrifuge:	Sorvall RC-5B, RC 26 plus, RC 28S with rotors SS34, GSA and GS3 (DuPont, USA)
	Centrifuge 5810 (R) (Eppendorf, Hamburg)
	Bench-top centrifuge 5415 and 5415 D (Eppendorf, Hamburg)
Electrophoresis:	Vertical gel electrophoresis apparatus (BioRad, Hercules, USA)
	Horizontal gel electrophoresis apparatus (Biometra, Göttingen)
	Power supply Phero-stap 500 (Biotec Fischer, Reiskirchen)
	Gel Doc 1000 (BioRad, Hercules, USA)
Thermal cycler:	Thermal Cycle GeneAmp PCR 9700 (PE Applied Biosystems,USA)
	Thermal Cycle 480 (Perkin Elmer, USA)
Shaking incubator:	Shaker (Duomax 2030, Heidolph)

DNA sequencer:	Nucleotide sequencer (ABI PRISM 3100-Avant Genetic analyzer, Hitachi)
UV spectrophotometer:	Photometer Ultrospec 3000 (Pharmacia, Uppsala, Sweden)
Others	Phospho Imager Typhoon 9410 (Amersham Biosciences, USA)
	Sonicator Sonorex RX-100 (Bandelin, Berlin)
	Balance BP 3100S (Sartorius, Göttingen)
	pH-Meter pH 526 (WTW, Weilheim)

6. Isolation of nucleic acid

6.1 Isolation of total RNA

Total RNA was isolated from young roots of *P. indica* plants as described by Salzman et al. (1999). The extraction buffer was composed of 4 M guanidine thiocyanate, 100 mM Tris-HCl, pH 8.0, 25 mM sodium citrate and 0.5 % (w/v) *N*-lauryl sarcosine. Polyvinylpolypyrrolidone (PVPP) (1 g) and 200 µl 2-mercaptoethanol were added to the buffer before use. Fresh sample (1 g) was frozen in liquid nitrogen and ground in a mortar into a fine powder. The powdered tissue was transferred into a SS34 centrifuge tube containing 10 ml extraction buffer and mixed strongly by shaking. Chloroform: isoamyl alcohol (24:1) (10 ml) was then added to the tissue solution, mixed and shaked vigorously for 10-20 min at room temperature. The insoluble debris was removed by centrifugation at 10,000xg for 10 min at 4 ºC in a fixed rotor (F28-50). The aqueous phase was transferred into a new SS34 tube, 10 ml of chloroform: isoamyl alcohol (24:1) was then added and mixed by vortexing for 30 sec. The chloroform mixture was separated by 10,000xg

centrifugation for 10 min at 4 °C, and the aqueous phase was transferred into a new SS34 tube. RNAs were precipitated by adding 0.1 volume of 5 M NaCl and 2 volumes of ethanol. After incubation at -20 °C for at least 3 h or overnight, the pellets were precipitated by centrifugation (10,000xg, 10 min at 4 °C), and dissolved in 10 ml sterile water. An equal volume of Tris-saturated phenol (pH 8.0) : chloroform : isoamyl alcohol (25:24:1) was added to the RNA solution, followed by shaking for 10 min at room temperature and centrifugation at 10,000xg for 10 min at 4 °C. The upper phase was carefully transferred to a clean tube, avoiding any interphase material. To the supernatant, 2 volumes of absolute ethanol and 0.1 volume of 5 M NaCl were added and the RNA was left to precipitate for at least 3 h or overnight. The solution was then centrifuged at 10,000xg for 10 min at 4 °C, and the pellet was resuspended in 500 µl of sterile distilled water. If quality was not adequate, the RNA was purified from protein impurities by LiCl precipitation. The sample was diluted to 1 ml with sterile distilled water, 333 µl of 8M LiCl were added, and RNA was precipitated at -4 °C for at least 3 h or overnight. The RNA pellet was recovered from the solution by centrifugation at 12,000xg for 20 min at 4 °C, washed with 400 µl of ethanol and then dried in vacuum. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer.

6.2 Isolation of poly A^+ mRNA

Poly A^+ mRNA was obtained from total RNA using Oligotex™ mRNA kit (Qiagen). The supplier's protocol was precisely followed. The principle of this method is based on the fact that most eukaryotic mRNAs (and some viral RNAs) end in a poly-A tail of 20-250 adenosine nucleotides. In contrast, rRNAs and tRNAs, which account for over 95% of cellular RNAs, are not polyadenylated.

Under conditions that include high-salt concentration, mRNA was purified by hybridizing the poly-A tail to a dT oligomer coupled to a solid-phase matrix. rRNA

and tRNA species do not bind to the oligo-dT and were eluted. The mRNA was released from the matrix by lowering the ionic strength and directly used for first strand cDNA synthesis for the cDNA library as described in section 7.

6.3 DNA isolation by agarose gels

DNA bands were separated by electrophoresis in 1x TAE containing 1.0% (w/v) agarose gel with ethidium bromide. After the desired DNA band was excised using a clean, sterile razor blade, the agarose slice was transferred to a microcentrifuge tube. The MinElute Gel Extraction Kit (Qiagen) was used to isolate the DNA from agarose gels. The principle of this procedure is the selective binding of DNA to a silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high chaotropic salt while contaminants pass through the column. Buffers provided with the kit enable 60-80% DNA recovery and removal of gel-based contaminants. The pure DNA is eluted with Tris-HCl buffer pH 8.0.

6.4 Isolation of plasmid DNA

Plasmid DNA was purified from 5-ml overnight cultures of *E. coli* cells in LB medium with appropriate antibiotic using QIAprep Spin Miniprep Kit (Qiagen). *E. coli* cells were pelleted by centrifugation at 4,000xg for 5 min. After the medium was removed, the pellet was resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube, then mixed with 250 µl of lysis buffer by inversion. Then, 350 µl of neutralization buffer were added. The solution was mixed by inversion, and centrifuged at 13,000 rpm for 10 min, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities were removed by a medium-salt wash. Plasmid DNA was eluted in high-salt buffer (10mM Tris-HCl, pH 8.5) or water. DNA concentration was measured using a spectrophotometer.

6.5 Quantitation of nucleic acids

The concentration of nucleic acids was determined photometrically at a wavelength of 260 nm. Depending on the class of nucleic acid, OD_{260}= 1.0 represents the following concentrations:

double-strand DNA	50	µg/ml
RNA	40	µg/ml

A sample containing nucleic acids was diluted with water (dilution factor) and the absorbance at 260 nm was determined with a spectrophotometer (Photometer Ultrospec 3000, Pharmacia). Water was used as a sample blank in the assay. The nucleic acid concentration was calculated by the following equation.

$$\text{DNA concentration } (\mu g/\mu l) = \frac{(OD_{260} \text{ x dilution factor x } 50\ \mu g)}{1000\ \mu l}$$

$$\text{RNA concentration } (\mu g/\mu l) = \frac{(OD_{260} \text{ x dilution factor x } 40\ \mu g)}{1000\ \mu l}$$

7. Cloning of a polyketide synthase cDNA from *P. indica*

The isolation of a polyketide synthase cDNA from *P. indica* was performed by Dr. Suphachai Samappito (Samappito, 2002) as follows.

7.1 Amplification of cDNA from *P. indica*

First-strand cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Life Technologies). To 10 µg of *P. indica* total RNA, 1.0 µl of oligo (dT) 12-18-primer were added and adjusted to 10 µl with water. The reaction mixture was then incubated for 10 min at 70 °C. After the mixture was chilled on ice, 4 µl of 5x reaction buffer, 2 µl of 2.5 mM dNTPs mix, and 2 µl of 100 mM DTT were added, and incubated at 37 °C for 5 min to allow the oligo (dT) primer to anneal to RNA template. Then, 200 U of M-MLV-reverse transcriptase were added, and the

volume of the reaction was adjusted to 20 µl with sterile water. After incubation at 37 °C for 1 h, the enzyme was inactivated by heating the reaction mixture to 94 °C for 5 min. A 1/10 volume of first-strand cDNA reaction mixture was used as cDNA template for RT-PCR in section 7.2.

7.2 Reverse transcriptase PCR (RT-PCR)

DNA fragments were amplified by mixing 100 ng of template cDNA as described in section 7.1. The following components were combined in PCR reactions:

10x PCR buffer	5 µl
$MgCl_2$	3 µl
dNTP mix (each 2.5 mM)	1 µl
cDNA	1 µl
upstream primer (PK 1 forward, 50 µM)	2 µl
5'-AA(A/G)GC(C/T)AT(A/C)GAIGA(A/G)TGGGG-3'	
downstream primer (PK 2.3 reverse, 50 µM)	2 µl
5'-CCACCIGG(A/G)TGI(A/G)CAATCC-3'	
DNA polymerase, 5 U/µl	0.5 µl
H_2O	added to 50 µl

Samples were subjected to 30 cycles of PCR, each incorporating 30 sec of denaturation at 94 °C, 30 sec of annealing at 48 °C, and 1 min of extension at 72 °C. The resulting DNA fragment was separated in 0.8% (w/v) agarose gel electrophoresis. The DNA fragment was isolated as described in section 6.3, ligated into pGEM –T Easy and sequenced. Sequencing of several clones confirmed that the PCR products showed similarity to a polyketide synthase.

7.3 Establishment of a cDNA library in λ-phages

Since it was presumed that plant polyketide synthases were expressed in *P. indica* roots, a *P. indica* cDNA library was screened for cDNAs encoding a plant polyketide synthase under low-stringency conditions. A ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) were used for the preparation of *P. indica* cDNA library.

7.3.1 Construction of λ-ZAP-cDNA Gigapack III library

Poly A^+ RNA was isolated from *P. indica* roots as described in section 6.2. *P. indica* cDNA library were synthesized following the Stratagene's protocol. The first strand cDNA was synthesized using Moloney Mouse Leukemia Virus (*M-MLV*) reverse transcriptase (37 °C, 1 h). The inclusion of 5'-methyl dCTP instead of dCTP during the first strand cDNA synthesis allowed the protection of cDNA from the host bacteria's restriction enzymes in the subsequent steps.

The second strand cDNA was synthesized by DNA polymerase I. First, mRNA that was hybridized to the first strand cDNA was nicked by Rnase H, resulting in a multitude of fragments that served as primers for the cDNA polymerase I to synthesize the second strand cDNA. During the second strand synthesis, the methyl-nucleotides were eliminated from the assay to allow the restriction with *Xho* I in a subsequent step. After the synthesis of second strand cDNA was completed, *Pfu* DNA polymerase was added to the assay to produce blunt 3'-and 5'-ends. The blunt 3'-and 5'-ends allowed a subsequent blunt-end ligation of *Eco*RI adapters.

The *Eco* RI adapter at the 5'-end was removed by *Xho* I restriction. This *Xho* I restriction resulted in a cDNA containing an *Xho* I sticky end at the 5'-end and an *Eco* RI sticky end at the 3'-end. The digested adapter as well as the free nucleotides were removed after size exclusion chromatography using a drip column

(Separose CL-2B gel filtration medium). The size-fractionated cDNA was precipitated. The cDNA was ligated into a Uni-ZAP vector and the ligated vector was packaged with a Gigapack III Gold Packaging extract. The resulting phage was used to infect the host bacteria.

A single colony of *E. coli* strain XL1-Blue MRF' was used to inoculate 50 ml of LB medium supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose. The bacteria were grown on a rotary shaker (200 rpm) at 37 °C for 6 h. The bacteria were harvested by centrifugation (500xg, room temperature, 1 min) and the pellet was resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. To determine the size of the phage cDNA library, the λ-ZAP cDNA library was titered in the following way: To 200 μl of host bacteria, 1 μl of diluted λ-phage was added (phage dilutions: 1:10 and 1:100 in SM buffer) and incubated at 37 °C to allowe the attachment of λ-phage to the host bacteria. After 15 min incubation, melted NZY top agar (3 ml; 48 °C) containing IPTG (final concentration: 2.5 mM) and X-Gal (final concentration: 4 mg/ml) was added and mixed. This mixture was immediately plated onto pre-warmed (37 °C) NZY-agar plates. The plates were incubated overnight at 37 °C. Plaques were counted on the following day. The percentage of cDNA inserts in the cDNA library was determined by a blue-white screening.

7.3.2 Screening of λ-ZAP-cDNA Gigapack III Library

The phage cDNA library, plated for screening, was prepared in a similar way as described in the previous section. To 200 μl of host bacteria resuspended in sterile 10 mM $MgSO_4$ to OD_{600} of 0.5, λ-phage was added and incubated at 37 °C for 15 min to allow phage to attach to the bacteria. Melted NZY top agar (6.5 ml for primary screening and 3 ml for subsequent screening; 48 °C) was added and mixed. This mixture was immediately plated on the pre-warmed (37 °C)

petri dishes containing NZY agar. Petri dishes of 150 mm in diameter were used for primary cDNA screening and petri dishes of 100 mm in diameter were used for the subsequent screening. The plaques developed after overnight incubation at 37 °C.

The following components were used in cDNA library screening:

20 x SSC buffer :	0.3 M sodium citrate/HCl, pH 7.0
	3 M NaCl
SM-buffer :	50 mM Tris-HCl, pH 7.5
	100 mM NaCl
	8 mM $MgSO_4$
	5 ml 2% (w/v) gelatin
Denaturation solution :	0.5 M NaOH
	1.5 M NaCl
Neutralization solution :	0.5 M Tris-HCl, pH 8.0
	1.5 M NaCl
Washing solution :	0.2 M Tris-HCl, pH 7.5
	2x SSC buffer

The screening was performed using nitrocellulose filters (ϕ132 mm; Sartorius; ϕ 82 mm; Schleicher & Schuell). The filters were put on the agar plates and the following procedure was followed:

Transfer from the plate to filter	2 min
Denaturation of filter in denaturation buffer	2 min
Neutralization of filter in neutralization solution	1 min
Additional neutralization of filter in neutralization solution	4 min
Filter washing with washing solution	0.5 min

The filters were subsequently air-dried and the transferred DNA was fixed on the filters by baking at 80 °C for 2 h. The filters with fixed DNA were

hybridized with [α-^{32}P]-labeled probe as described previously (Samappito, 2002). The phages of interest were localized on the NZY agar petri dishes using autoradiograms. The agar containing the plaques of interest was cut out and the phage was eluted from the agar in 500 µl SM buffer supplemented with 20 µl $CHCl_3$. After the content of the tube was vortexed and centrifuged (500xg, room temperature, 1 min), the phage-containing supernatant was diluted in SM buffer (ratio 1:100) and used for the subsequent screening steps or for *in vivo* excision.

7.3.3 *In vivo* excision

E. coli strain XL1-Blue MRF' and *E. coli* strain SOLR were grown overnight in LB medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ at 30 °C. The next day, cells were centrifuged (1000xg, 1 min, room temperature) and resuspended in sterile 10 mM $MgSO_4$ to an OD_{600} of 1.0. The following components were transferred to a 10 ml polypropylene tube: 200 µl of *E. coli* XLI-Blue MRF' cells in $MgSO_4$, 200 µl of isolated phage and 1 µl ExAssist helper phage. After 15 min incubation at 37 °C (phage attaching to bacteria), 3 ml of LB broth were added to the tube. The bacteria were cultivated for 3 h at 37 °C to enable the excision of pBluescript phagemid from the phage. A heating step (70 °C, 20 min) followed to inactivate the bacteria. The bacteria were centrifuged (1000xg, 15 min, room temperature) and the supernatant containing the excised phagemid was stored at 4 °C.

The medium with the excised phagemid (1 µl) was combined with 200 µl of *E. coli* SOLR cells, diluted in sterile $MgSO_4$. This strain contains an amber mutation that prevents the replication of phage genome, but allows the replication of phagemid. The bacteria were incubated for 15 min at 37 °C and plated on LB agar plates supplemented with ampicillin (50 mg/l). The bacteria were grown overnight

(37 °C). The next day, single bacterial colonies were transferred to LB supplemented with ampicillin (50 mg/l). The phagemids were isolated from the bacteria and were analyzed for the occurrence of inserts by restriction digestion with *Eco*RI and agarose gel electrophoresis.

8. Enzymatic modifications of nucleic acids

8.1 Restriction analysis

Bacterial restriction endonuclease enzymes are able to specifically hydrolyze DNA after recognition of a specific sequence in double-stranded DNA. These enzymes represent a useful tool to cut the desired fragments from DNA-molecules. The restriction endonucleases from Life Technologies and New England Biolabs were used. DNA digestion was incubated at 37 °C for more than two hours and the standard reaction conditions were:

DNA	1-10 µg
10 x reaction buffer	2 µl
restriction endonuclease enzyme	1 U
sterile water	to final volume of 20 µl

The resulting digested DNA was used for further ligation with plasmid.

8.2 Ligation reaction

The enzyme T4 DNA ligase catalyzes the formation of a phosphodiester bond between the 5'-phosphate and the 3'-hydroxy end of double-stranded DNA molecules. T4 DNA ligase from Promega (4U/µl) was used in the following standard ligation reaction:

Vector (pGEM-T Easy)	10-20 ng
Insert DNA	50 ng
10 x buffer T4 DNA ligase	2 µl
T4 DNA ligase	4 U
Sterile water	to final volume of 10 µl

A molar ratio of insert to vector of 3:1 was applied in all ligation reactions. The quantity of the insert was calculated with the formula:

$$\frac{\text{ng vector} \times \text{kb insert size} \times (\text{molar ratio insert:vector})}{\text{Kb vector size}} = \text{ng insert}$$

The ligation reactions were performed in eppendorf reaction tubes. The tubes were incubated in a cooling water bath at 16 °C overnight. Ligated vectors were subsequently transformed into competent bacteria as described in section 9.

9. Transformation of competent *E. coli* cells

A heat-shock method is a simple and an efficient transformation method when small vectors (up to 10 kb in size) are used for transformation. For each transformation, the contents of tubes containing 100 µl of competent cells were slowly melted on ice. The product of a ligation reaction (10 µl) (section 8.2) was pipetted to the cells and incubated for 30 min on ice. The transformation occurred during a heat shock (5 min in water bath of 42 °C), then immediately transferred to ice (2 min incubation) and 0.5 ml LB medium were added to the cells. The cells were incubated for 45 min at 37 °C and were then transferred to the petri dishes containing LB agar plus ampicillin. The transformed bacteria were grown overnight at 37 °C, and single colony was used for further analysis.

10. Analysis of transformant by PCR

To analyze transformants which contained the correct insert, colony PCR with vector-specific primers was performed. A single colony was picked from an agar-plate and transferred into a PCR tube containing 50 µl LB medium containing the appropriate antibiotic. The PCR tube was incubated at 37 °C for 5 h with shaking at 180 rpm. Subsequently, the PCR reaction was performed in 50 µl total volume as follows:

H_2O	36.5 µl
10x Taq buffer with 15 mM $MgCl_2$	5 µl
10 mM dNTPs	1 µl
10 µM sense primer	1 µl
10 µM antisense primer	1 µl
Tag polymerase	0.5 µl
Bacterial culture	5 µl
Total volume	50 µl

The vector specific primers for the cloning vector (pGEM-T Easy, Promega) were T7 primer: 5'-GAATTGTAATACGACTCACTATAG-3' as sense primer and SP6 primer: 5'-GATTTAGGTGACACTATAGAATAC-3' as antisense primer. The following PCR program was used to screen for white colonies: 3 min at 94 °C for 1 cycle, then 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 90 sec. The last step was followed by an additional elongation step at 72 °C for 7 min. The PCR products were analyzed by 1% agarose gel electrophoresis. Positive clones containing an insert of 1.2-kb were then sequenced.

Vector specific primers for the expression vector (pET14b, Novagen) were *Nde*I primer and *Bam* HI primer as described in section 11. The following PCR program was used for analysis of transformants: 5 min at 94 °C for 1 cycle, then 30 cycles of

94 °C for 30 sec, 55 °C for 1 min, 72 °C for 3 min. The last step was followed by an additional elongation step at 72 °C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis. The positive clones containing an insert of approximately the size of the PCR product were then sequenced.

11. Construction of expression vector for PinPKS full-length gene

To express the cDNA in *E. coli*, the open reading frame was cloned into a pET14b vector (Novagen), which contains a hexahistidine N-terminal fusion tag. The PinPKS full-length cDNA was amplified by PCR using 5' *Nde*I primer: 5'-ATTTTTCATATGGCACCAGCAGTTCAA-3' as forward primer (the *Nde*I site is underlined) and 5' *Bam*HI primer: 5'TTTAAAGGATCCTTAGTTAAGCGGCACACT-3' as reverse primer (the *Bam*HI site is underlined) by *Pfu* DNA polymerase (Promega) (Samappito, 2002). The following PCR program was used for the DNA amplification: 3 min at 94 °C for 1 cycle, then 25 cycles of 94 °C for 3 min, 48 °C for 1 min, 72 °C for 30 sec. The last step was followed by an additional elongation step at 72 °C for 7 min. The 1.2-kb PCR product was digested with *Nde*I and *Bam*HI at 37 °C overnight, gel-purified and ligated into *Nde*I/*Bam*HI–digested pET14b to generate the expression plasmid. *PinPKS*:pET-14b was subsequently transformed into a cloning *E. coli* strain (*E. coli* DH5α). The plasmids were isolated from bacteria and the occurrence of inserts in the plasmids was investigated by a restriction endonuclease digestion using *Nde*I/*Bam*HI. The expression vector constructed was transformed into the expression host *E. coli* BL21 (DE3). Heterologous expression and the preparation of crude bacterial extracts were performed as described in section 12.

12. Expression and purification of recombinant proteins

The following buffers were used during the expression and purification of recombinant proteins:

His-tag lysis buffer (HLB):	50 mM Tris-HCl, pH 7.0
	500 mM NaCl
	2.5 mM imidazole
	10 mM β-mercaptoethanol
	10% (v/v) glycerol
	1% (v/v) tween 20
	750 μg/ml lysozyme
His-tag wash buffer (HWB):	50 mM Tris-HCl, pH 7.0
	500 mM NaCl
	2.5 mM imidazole
	10 mM β-mercaptoethanol
	10 % (v/v) glycerol
His-tag elution buffer (HEB):	50 mM Tris-HCl, pH 7.0
	500 mM NaCl
	50 mM imidazole
	10 mM β-mercaptoethanol
	10 % (v/v) glycerol
Enzyme storage buffer:	100 mM HEPES, pH 7.0
	10% (v/v) glycerol

12.1 Bacterial culture and expression of recombinant protein

The *E. coli* strain BL21 (DE3) transformed with expression vectors PinPKS: pET-14b (see section 11) was streaked onto LB agar plate containing

ampicillin (50 mg/l) and incubated at 37 °C overnight. A single colony was transferred and cultured in 50 ml LB medium containing 50 µg/ml ampicillin at 37 °C overnight with shaking at 180 rpm as a starter culture. The starter culture was transferred to a flask containing 950 ml of pre-warmed LB broth containing 100 µg/ml ampicillin. The bacteria were cultivated on a rotary shaker (180 rpm) at 37 °C until the OD_{600} had reached 0.6. The flask was cooled on ice for 5 min and IPTG was added in required amounts to induce the protein expression. The final IPTG concentration for the bacteria transformed with pET-14b vector constructed was 0.4 mM. The expression of *Pin*PKS recombinant protein was allowed to proceed at 18 °C overnight with shaking at 180 rpm. The induced cultures were chilled on ice and the bacteria were harvested by centrifugation at 8,000 rpm for 20 min at 4 °C. The supernatants were decanted and the pellets were frozen at -80 °C.

12.2 Purification of recombinant proteins

Frozen *E. coli* cells were resuspended in 20 ml HLB buffer. The cell suspension was incubated for 1 h on ice to allow the lysis to proceed. Subsequently, the suspension was sonicated (pulse sonication; 2 x 30 sec) and centrifuged (20 min, 12,000xg, 4 °C). The supernatant containing the heterologous protein was decanted into a new tube.

TALON Metal Affinity Resin (Clontech) was used for purifying recombinant polyhistidine-tagged proteins. TALON Resin was equilibrated with HWB buffer prior to the addition of supernatant according to the supplier's instructions. TALON resin (2 ml) were transferred to a 15 ml polypropylene tube, centrifuged (700xg, 2 min, 4 °C) and washed twice with 10 ml HWB buffer. The bacterial supernatant containing the recombinant protein was applied to the equilibrated TALON resin and was gently shaken for 60 min to allow the binding of his-tagged recombinant protein to the resin. The resin was subsequently centrifuged (700xg, 5 min, 4 °C), resuspended in 10 ml

washing buffer (HWB) and agitated again for 10 min at room temperature. The washing step was repeated once. Following centrifugation (700xg, 5 min, 4 ºC), the resin was resuspended in 2 ml HWB buffer and transferred to an end-capped 2 ml gravity flow column (Clontech). The resin was allowed to settle. After the resin had settled, the end cap was removed to allow the buffer to drop from the column. The resin was washed with 10 ml HWB buffer. The protein that bound to the resin was eluted with 5 ml HEB buffer. Fractions (1 ml) were collected. The protein content of individual fractions was determined by the Bradford assay (Bradford, 1976).

To remove low molecular weight substances (especially imidazole and NaCl), the fractions containing recombinant protein were combined to a total volume of 2.5 ml and applied to a PD-10 column (Amersham Biosciences) that had been previously equilibrated with 25 ml of enzyme storage buffer. After the protein fraction entered the PD-10 column, 4 ml of enzyme storage buffer were added to the column for elution and 1 ml fractions were collected. The purity of collected fractions 2 and 3 containing recombinant protein were determined by electrophoresis on denaturing polyacrylamide gel as described in section 15. The protein concentration of the purified enzyme was determined by the Bradford assay and used for polyketide synthase assay.

12.3 Determination of protein using a Bradford assay

Protein concentration was determined using a Bradford assay (Bradford, 1976). This assay is based upon unspecific binding of Coomassie Brilliant Blue G-250 to proteins in acidic solution. A shift of absorbance-maximum from 465 nm to 595 nm can be photometrically determined. Bovine serum albumin (Sigma) was used as standard.

A sample with unknown protein concentration was dissolved in 800 µl H_2O Two hundred milliters of BioRad Protein Assay (BioRad, München) were added

to the sample solution. After 5 min incubation at room temperature, the absorbance at 595 nm was determined. The protein concentration in the sample was determined using a bovine serum albumin standard curve.

13. DNA sequencing

13.1 Big Dye Terminator sequencing reaction

The reaction was performed in a volume of 10 µl containing 0.5 µl of Big Dye cycle sequencing mix, 1.75 µl of 5x buffer, 1 µl of 5-10 mM primer and plasmid DNA (600 ng). The PCR amplification was carried out by the following program: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. Subsequently, 10 µl of sterile water was added to the PCR reaction mixture.

13.2 Purification of Big Dye reaction mixture

The product of the Big Dye reaction was purified from the reaction mixture prior to sequencing. Sephadex G50 Superfine (Pharmacia) was loaded into the wells of a MultiScreen^R HV plate by using a column loader and then 300 µl of sterile Milli Q water were pipetted into each well. The plate was covered and the gel was allowed to swell at 4 °C for more than 3 h or overnight. The water was removed from the column by centrifugation at 910x g for 5 min at 4 °C. Then, the Big Dye reaction was loaded drop by drop into the center of each column. The plate with the gel filtration columns was then placed on the top of 96-well microtiter plate and centrifuged at 910x g for 5 min at 4 °C. The eluted DNA sample was ready for sequencing using the ABI PRISM 3100-Avant Genetic analyzer (Hitachi).

14. Sequence analysis

The nucleotide sequences were analyzed using DNASTAR software and translated into amino acid sequences. The sequence comparison was performed using BLAST search. This software is available on the internet at http//www.ncbi.nlm.nih.gov or www.ddbj.nig.ac.jp.

15. Gel electrophoresis

15.1 Agarose gel electrophoresis

DNA analysis was performed using 1 % agarose gels containing 1xTAE buffer:

1 x TAE buffer:	40 mM Tris
	20 mM CH_3COOH
	1 mM EDTA

Agarose gels (volume of 50 or 100 ml) were prepared as follows: Agarose was melted in 1x TAE buffer in a microwave oven. Ethidium bromide (0.4 µg per 1 ml gel) was added to the gel when the solution had cooled down to about 65 ºC. The gel was immediately poured into horizontal trays for the agarose gel electrophoresis. The hardened gel was transferred to the gel electrophoresis chamber containing 1x TAE buffer. DNA samples were mixed with 5x loading buffer (40% (w/v) Saccharose 0.001% (w/v) Orange G). The samples were pipetted into the gel pockets and run at 75 Volts for about 1 h. Separated DNA was visualized by exposing to UV light and the gels were photographed using the Gel Doc Equipment (Gel Doc 1000, BioRad).

15.2 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels (10%) were used for analysis of protein mixture, monitoring protein purification and determining the relative molecular mass of proteins. The acrylamide stock solutions ((30% (w/v) acrylamide with 0.8% (w/v) bisacrylamide) were obtained from Roth (Karlsruhe). The following components were needed to perform an SDS-PAGE electrophoresis.

10% SDS-PAGE	4.1 ml H2O
(separation gel):	2.5 ml 1.5 M Tris-HCl, pH 8.8
	3.3 ml acrylamide/ bisacrylamide
	50 µl 20% SDS
	5 µl TEMED (Roth)
	50 µl 10% ammonium persulfate (APS)
4% SDS-PAGE	3.075 ml H_2O
(stacking gel):	1.25 ml 0.5 M Tris-HCl, pH 6.8
	670 µl acrylamide/ bisacrylamide
	25 µl 20 % SDS
	5 µl TEMED
	25 µl 10 % APS
5x running buffer (pH 8.8):	15 g Tris, 72 g glycine, 5 g SDS
	Dissolved in one liter H_2O
5x Sample buffer:	0.25 M Tris-HCl, pH 6.8
	6% (w/v) SDS
	8% (v/v) glycerol
	0.02 % (w/v) bromophenol blue

The separating gel was mixed, passed through a 0.45 µM filter and poured between two glass plates (100 mm x 73 mm; 100 mm x 83 mm). A distance of 0.5 mm between plates was assured by appropriate spacers. The top of the gel was covered with *n*-butanol-water to allow the polymerization. After gel polymerization for 1 h, *n*-butanol was removed and the stacking gel was added. A comb was inserted to allow the formation of loading pockets. After the gel polymerized, it was transferred together with the glass plates to the electrophoresis apparatus that contained the running buffer. For sample preparation, 7 µl of sample buffer and 7 µl of DTT (8 mg/ml) were added to 10 µl of protein solution and mixed. The sample was denatured at 95 °C for 5 min and placed on ice for 1 min. After centrifugation to precipitate insoluble matters, samples were loaded on the gel and run at 80 Volts for about 2 h.

Following the electrophoresis, Coomassie blue staining was performed. The SDS gel was fixed and stained in a staining solution as follows:

Staining solution:	0.1 % (w/v) Coomassie Brilliant Blue R-250
	40 % (v/v) methanol
	10 % (v/v) acetic acid
Destaining solution:	40 % (v/v) methanol
	10 % (v/v) acetic acid

After at least 1 h of staining, the gel background was removed in a destaining solution by gently shaking for more than 1 h. The gel was stored in the solution containing 1% (v/v) acetic acid and 3% (v/v) glycerol.

16. Polyketide synthase assay

16.1 Standard assay of recombinant enzyme

The standard enzyme assay contained 20 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA (80,000 dpm), 1 mM NADPH, 200 mM potassium phosphate buffer, pH 6.0 and 10 µg of recombinant enzyme in a final volume of 100 µl. The assay mixture was incubated at 30 °C for 2 h and the reaction mixture stopped by adding 10 µl of 10% (v/v) HCl. The enzymatic products were extracted twice with 200 µl of ethyl acetate and separated by TLC. TLC was performed on a RP18 plate (Merck) developed in methanol: H_2O: acetic acid (75:25:1) or a silica gel 60 F254 plate (Merck) developed either in ethyl acetate: methanol: H_2O (100:16.5:13.5) or toluene: formic acid (99:1). The radioactive products were visualized by phosphorimaging (Typhoon 9410, Amersham Biosciences). Reaction products were identified by the use of authentic standards as well as comparison to published profiles of chalcone synthase from *Cassia alata* (CalCHS1) (Samappito et al., 2002).

16.2 Coupled assay of recombinant enzyme and crude protein extract

Crude protein extracts of *P. indica* tissue cultures (from section 4.1) were incubated with recombinant PinPKS. The reaction mixture contained 200 mM potassium phosphate buffer, pH 6.0, 20 µM acetyl-CoA, 70 µM [2-^{14}C] malonyl-CoA (80,000 dpm), 1 mM NADPH, 10 µg of recombinant enzyme and 25 µl of the crude protein extracts prepared from each *P. indica* tissue culture in a final volume of 100 µl. The assay mixture was incubated at 30 °C for 2 h and the reaction stopped by adding 10 µl of 10% (v/v) HCl. The enzymatic products were extracted twice with 200 µl of ethyl acetate and separated by TLC. TLC was performed on a silica gel 60 F254 plate (Merck) developed in toluene: formic acid (99:1). The radioactive products

were visualized and the radioactivity determined by phosphorimaging (Typhoon 9410, Amersham Biosciences).

17. Analytical methods

17.1 Analysis of enzymatic products by HPLC

The enzymatic products from the PinPKS assay and the PinPKS assay coupled with crude protein extract of *P. indica* were analyzed by HPLC and radio-HPLC. Ten times of the scaled-up of 500 µl reactions were performed. The reaction mixture contained 150 µM acetyl-CoA, 350 µM malonyl-CoA, 5 mM NADPH and 40 µg of recombinant enzyme in 200 mM potassium phosphate buffer, pH 6.0. Crude protein extracts obtained from *P. indica* root culture (150 µl) was added to the incubation mixture of a coupled assay. For radio-HPLC analysis, the reaction used acetyl-CoA and [2-^{14}C] malonyl-CoA as substrates. The reaction mixture was incubated at 30 °C for 2 h and stopped by adding 50 µl of 10% (v/v) HCl. The enzymatic products were extracted twice with 500 µl of ethyl acetate. After flushing with N_2 gas, the residue was dissolved in methanol and injected onto a Lichrospher 60, RP-select B column (250 x 4 mm, 5 µm) (Merck) connected with Hewlett Packard Series 1100 HPLC. The solvent systems were (A): 98% (v/v) H_2O, 2% (v/v) acetonitrile and 2% (v/v) acetic acid and (B): 98% (v/v) acetonitrile, 2% (v/v) H_2O and 2% (v/v) acetic acid, with the following gradient: 0-25 min 0-60% B, 25-30 min 60% B, 30-32 min 60-100% B, 32-35 min 100%, 35-37 min 100-0% B and 37-40 min 0% B at a flow rate of 1 ml/min. The enzymatic products were monitored at 280 nm.

17.2 Analysis of enzymatic products by LC-ESI-MS

To identify by LC-ESI-MS the enzymatic products from the PinPKS assay and the PinPKS assay coupled with crude protein extract of *P. indica*, Twenty times of the scaled-up of 500 µl reactions were performed. The reaction mixture contained 150 µM acetyl-CoA, 350 µM malonyl-CoA, 5 mM NADPH and 100-150 µg of recombinant enzyme in 200 mM potassium phosphate buffer, pH 6.0. Crude protein extracts obtained from *P. indica* root culture (150 µl) was added to the incubation mixture of a coupled assay. The reaction mixture was incubated at 30 °C for 2 h and stopped by adding 50 µl of 10% (v/v) HCl. The enzymatic products were extracted twice with 500 µl ethyl acetate. After flushing with N_2 gas, the residue was dissolved in methanol and analyzed by LC-ESI-MS. The positive and negative ion electrospray (ESI) mass spectra of the enzymatic products were obtained from a Finnigan MAT TSQ Quantum Ultra AM system equipped with a hot ESI source (HESI, electrospray voltage 2.5 kV, sheath gas: nitrogen; vaporizer temperature: 150 °C; capillary temperature: 250 °C; collision gas: argon; collision pressure: 1.5 mTorr). The MS system was coupled with a Surveyor Plus micro-HPLC (Thermo Electron) and equipped with an Ultrasep ES RP18E-column (5 µm, 1x100 mm, SepServ). For the HPLC a gradient system was used starting from H_2O:CH_3CN = 90:10 (each containing 0.2% acetic acid) to 10: 90 within 15 min; flow rate 50 µl/min. All mass spectra were averaged and background subtracted. The ESI-CID mass spectra were obtained during the HPLC run with a collision energy of 15 eV (negative ions) and -20 eV (positive ions), respectively.