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Table: 1, Figs : 5

Cloning, expression and wounding induction of β-caryophyllene synthase gene from *Mikania micrantha* H.B.K. and allelopathic potential of β-caryophyllene

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ABSTRACT

β-Caryophyllene is an important volatile sesquiterpene of plants that may serve as allelochemical to influence the neighboring plant growth or as an indirect defence to attract natural herbivore enemies. A partial cDNA for β-caryophyllene synthase gene was isolated from the expressed sequence tag (EST) library of *Mikania micrantha* leaves. The full length cDNA of β-caryophyllene synthase from *M. micrantha*, designated as *MmCS* was obtained by rapid amplification of cDNA ends (RACE) methods. This *MmCS* cDNA is 1898 bp in full length and it encodes a putative protein of 547 amino acids. *MmCS* expression was significantly increased in *M. micrantha* leaves within 3-days after wounding during a 5-day interval following mechanical wounding. Bioassay showed that β-caryophyllene at ≥ 3 mg L^{-1} significantly inhibited the germination rates and seedling growth of *Brassica campestris* and *Raphanus sativus*. These results suggest that β-caryophyllene synthase and β-caryophyllene may play an important role in allelopathy for successful invasion of *M. micrantha*.

Key words: Allelopathy, *Brassica campestris*, β-caryophyllene, β-caryophyllene synthase gene, *Mikania micrantha*, *Raphanus sativus*, wounding.

INTRODUCTION

Mikania micrantha H.B.K. (Compositae), a perennial vine native to South and Central America, is one of the worst invasive weeds in the world (18). It was brought to South China in 1910 and its invasion has now caused substantial damage to natural ecosystem and biodiversity in Guangdong province (19,26). Recent studies have shown that the successful invasion of *M. micrantha* is not only due to its high reproduction (12) and wide eco-physiological tolerance (25), but also due to its allelopathic effects on neighbouring native plants (14,19,20).

Allelochemicals of *M. micrantha* are released by decomposition of plant debris or volatilization (19). Volatile oil of this plant significantly inhibited the growth of various plants and pathogenic fungi (27,29). The volatile oil from *M. micrantha* flowers contains high concentrations of α-pinene and β-pinene, both of which are effective insect repellents

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(11). The volatile oil of *M. micrantha* had strong deterrent effect on oviposition of *Plutella xylostella*, *Phyllotreta striolata* and *Phaedon brassicae* (28).

Terpenoids are a diverse group of secondary compounds with a variety of functions, playing an important role in plant-plant, plant-insect and plant-pathogen interactions (2,4,21). Sesquiterpenes are a large family of C_{15}-iso-prenoids. β-Caryophyllene is common sesquiterpene, widely distributed in plants and has allelopathic potential (17). β-Caryophyllene synthase catalyses the conversion from farnesyl diphosphate to β-caryophyllene (3). β-Caryophyllene inhibits the development of crops seedlings viz., radish (*Raphanus sativus* L.), mung bean (*Vigna radiata* (L.) Wilczek) and tomato (*Lycopersicon esculentum* Mill.) (16). Kil *et al*. (15) reported that β-caryophyllene was important component of essential oil of *Artemisia lavandulaefolia*, which suppressed the seedling growth of *Achyranthes japonica* (Miq.). β-Caryophyllene also exists in *M. micrantha*, stems and leaves (9.49%) and flowers (9.17%) (10,23).

To better understand the relationship between invasive plants success and allelopathic potential of volatiles of *M. micrantha*, we constructed the cDNA library from *M. micrantha*, cloned β-caryophyllene synthase cDNA and characterized its expression profiles.

METHODS AND MATERIALS

M. micrantha plants at vegetative stage approximately 120 cm tall were collected in October, 2006 from a population at Qi Ao Island, Zhuhai, China (N 21°48′, E 113°3′). They were cut into 10 cm long pieces and transplanted into plastic pots (25 cm dia; 20 cm height) in a green house (10 h light and 14 h dark at 25±1°C, 75±5% relative humidity) on campus in Guangzhou, China. The plants were allowed to climb on 100 cm bamboo sticks and watered with diluted Hoagland solution (25% v/v) until harvest for experiments. Seeds of *Brassica campestris* and *Raphanus sativus* were purchased from Seed Company, Guangzhou, China.

RNA extraction

Total RNA was isolated from the leaves of *M. micrantha* using the guanidine thiocyanate method (6), further purified using silica particles as per Ding *et al.* (8) and was quantified by absorbance at 260 nm. The integrity of the RNA was checked using 0.35% agarose gel electrophoresis.

Cloning of full-length *MmCS* cDNA

The double-stranded cDNA was synthesized using SMART ™ cDNA amplification Kit (Clontech). PCR products were purified using QIAquick PCR purification kit (Qiagen), then inserted into pGEM®-T easy vector (Promega) and finally transformed into *E. coli* Top10 (Invitrogen). The positive clones were sequenced by ABI 3730.

To obtain the full-length cDNA of β-caryophyllene synthase of *M. micrantha*, designated as *MmCS*, we designed specific primer WGJ87R2 (5′-CCAAACGTCCTCAAT-TTCCTCCCTC-3′) according to the EST sequences of WGJ87. The adapter primers ZF407 (TS-long primer, 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAA-

CGCAGAGT-3′), ZF408 (TS-short primer, 5′-CTAATACGACTCACTATAGGGC-3′) and ZF409 (nested TS-PCR primer, 5′-AAGCAGTGGTATCAACGCAGAGT-3′) were used to perform 5′ RACE.

Sequence analysis

Multiple sequence alignment of β-caryophyllene synthase cDNA from *M. micrantha* and other β-caryophyllene synthases were performed with Clustal X 1.83. Homologies were calculated with the MegAlign of DNAStar Software. Phylogenetic tree was constructed using MEGA 4.0.

Induction of the *MmCS* by wounding

Wounding of basal leaves of *M. micrantha* was done in the same condition as described above. The fourth and fifth leaves (counted basipetally from the apex) of the plants (about 50 cm tall) grown in the greenhouse were mechanically wounded with scissors. There were two cut in each leaf. Wounded plants of each group were harvested at 1, 2, 3, 4 and 5d, respectively, after the wounding. All harvested samples were immediately placed into liquid N_2 for RNA extract.

Semi-quantitative RT-PCR analysis of *MmCS*

Total RNA was isolated from different tissues of plants with the same method described above. The RNA was first treated with DNase I and then reverse transcribed using M-MLV Reverse Transcriptase with oligo (dT) primer as per the manufacturer's instructions. The ubiquitin gene was chosen as internal standard. The integrity of all RNA samples was further verified by the successful amplification of ubiquitin. The PCR primers were WGJ87F1 (5′-TAAGAAGGAGCAAGAAAGAGTGC-3′) and WFG87R1 (5′-CTCTTTGATGTCTTCTTCCACTTC-3′) for *MmCS*, which generated a PCR product of 259 bp; WGJ60 (5′-GATTCCACCAGACCAGCAAAGG-3′) and WGJ61 (5′-CACCACG-AAGACGAAGCACAAG-3′) for ubiquitin, which generated a PCR product of 122 bp. To ensure the semi-quantitative nature of this measurement, the first-stand cDNA was firstly tested for different cycle numbers ranging from 20 to 28 for ubiquitin and from 26 to 34 for *MmCS*. The PCR products (3μl) were separated on 1.5% agarose gel and visualized by the ethidium bromide staining with UV illumination. The intensity of each band was analyzed with GelDoc2000 system and Molecular Analyst Software (Bio-Rad, CA, USA). The optimal numbers of PCR cycles were 22 for ubiquitin and 30 for *MmCS* expression in different samples. The cycling conditions were 94°C for 0.5 min, 58°C for 0.5 min, 72°C for 0.5 min, and a final extension at 72°C for 10 min. All PCR products were analyzed as described above.

Bioassay of β-caryophyllene

To investigate the allelopathic potential of β-caryophyllene, *B. campestris* and *R. sativus* were chosen as the test plants. Their seeds were surface-sterilized with 0.5% $KMnO_4$ for 15 min and then washed with sterile water. β-Caryophyllene (> 98.5%) obtained from Sigma-Aldrich was dissolved in ethyl acetate and then different volumes of this solution were added to filter paper in glass pots (6×6×10 cm) to get 0.375, 3 and 24 mg L^{-1} concentrations. After complete evaporation of ethyl acetate, 20 seeds of each test species and 5 ml distilled water were placed in each glass pot. Plants were grown in

greenhouse (at 25±1°C with 10 h light and 14 h dark). The germination rate was recorded at 3 d after incubation and seedling root length and shoot height were recorded at 7 d. Seeds with minimal root length of 1 mm were considered as germinated seeds. All experiments were repeated three times independently.

The magnitude of inhibition or stimulation in bioassay was denoted as the response index (RI) and was calculated as under:

$$RI\ (\%) = (T/C\text{-}1) \times 100$$

Where, T: Treatment data, C: Control data.

Statistical analysis: The expression levels of *MmCS* in each sample were calculated as the ratio to that of ubiquitin in semi-quantitative RT-PCR analysis. All data were presented as mean ± SD. Significant differences ($P<0.05$) were analyzed using one-way ANOVA followed by Duncan's multiple range test using SPSS 11.5 Software Package (SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Isolation and characterization of *MmCS*

A total of 783 clones were isolated and sequenced from the expressed sequence tag (EST) library of *M. micrantha* leaves. Among these clones, one cDNA (named WGJ87, 658 bp) with the 3′ -terminal poly (A) sequence showed high homology with the β-caryophyllene synthase from *Artemisia annua* (GenBank AF472361) (3). A full length cDNA of *MmCS* was further obtained by rapid amplification of cDNA ends (RACE) methods. The *MmCS* cDNA is 1898 bp in length (GenBank FJ767894), including the 3′-terminal poly (A) sequence. The sequence contains 57 bp 5′-UTR and 197 bp 3′-UTR. The open reading frame of *MmCS* cDNA consists of 1644 bp, coding for a protein of 547 amino acids. Sequence alignment showed that *MmCS* contained the GVYxEP element common to angiosperm sesquiterpene. The DDxxD divalent metal ion-substrate binding motif that was highly conserved in terpene synthases was also found in *M. micrantha* (Fig. 1) (3,7).

The plant terpene synthases family was divided into seven subfamilies by alignment of the amino acid sequence of terpene synthases, designated TPSa through TPSg (4,9). Alignment of the amino acid sequence of *MmCS* was devoid of the diterpene synthase insertion element and N-terminal organelle targeting information, so the *MmCS* belongs to the Tpsa subfamily of terpenoid sythases (3,24).

Homology analysis should that *MmCS* shared the moderate amino acid identities with β-caryophyllene synthase of other plants: 57.8% with *A. annua* (AAL79181), 36.4% with *Cucumis sativus* (AAU05952) but low identities with β-caryophyllene synthase of other plants: 30.7% with *Arabidopsis thaliana* (AAO85539), 29.3% with *Oryza sativa* (ABJ16553) and 26.9% with *Zea mays* (ABY79213). Therefore, *MmCS* differed from a basal node shared by β-caryophyllene synthase of *A. annua* (Fig. 2).

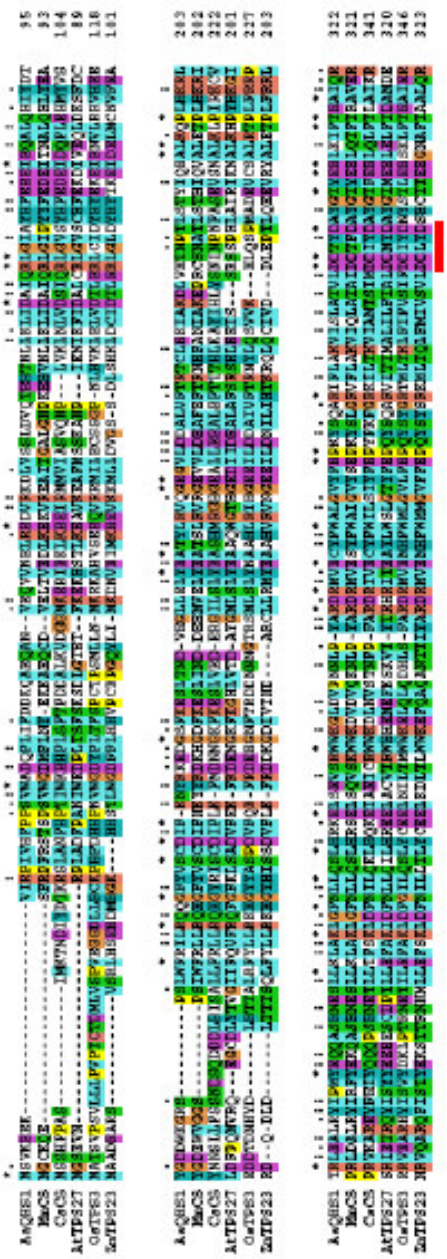

Figure 1. Comparison of the deduced amino acid sequence of *Mm*CS with other β-caryophyllene synthases from other plants. *Arabidopsis thaliana* AtTPS27, GenBank AAO85539; *Cucumis sativa* CsCS, AAU05952; *Artemisia annua* QHS1, AAL79181; *Zea mays* ZmTPS23, ABY79213; and *Oryza sativa* OsTPS3, ABJ16553. The highly conserved DDxxD region was marked with a line.

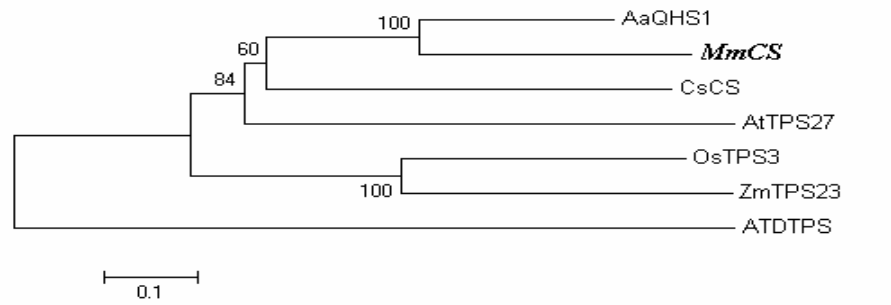

Figure 2. Phylogenetic analysis of the aligned amino acid sequence of *MmCS* with other β-caryophyllene synthases. The tree was constructed by the neighbor-joining method using MEGA 4.0. Values of the bootstrap support of the particular branching calculated for 10,000 replicates are indicated at the noted. Bootstrap values greater than 60% are indicated at the branch. The bar shows the branch length. ATDTPS indicates ent-kaurene synthase from *Arabidopsis thaliana*, a functionally different diterpene synthase that was used as an out group. Abbreviations and Genbank accession number: AaQHS1 (*A. annua*, AAL79181); AtTPS27 (*A. thaliana*, AAO85539); CsCS (*C. sativu,* AAU05952); OsTPS3 (*O. sativa*, ABJ16553); ZmTPS23 (*Z. mays*, ABY79213); *MmCS* (*M. micrantha,* FJ767894) and ATDTPS (*A. thaliana*, NP_178064).

Tissue distribution of *MmCS*

It is generally believed that β-caryophyllene synthase gene is expressed in most plant tissues during the early development period (3). To determine the expression pattern of *MmCS*, several tissues of *M. micrantha* were analyzed by RT-PCR (Fig. 3). *MmCS* transcripts were observed in most tissues except seeds, these were more in leaves (mature or young) and very less in the petioles and the flowers.

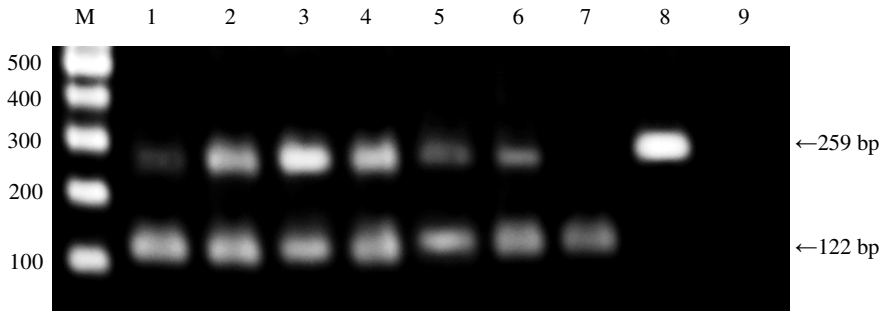

Figure 3. RT-PCR analysis of β-caryophyllene synthases transcripts in tissues of *M. micrantha*. Lane M is a DNA molecular marker. Total RNA was extracted from roots (1), stems (2), mature leaves (3), young leaves (4) petioles (5), flowers (6), seeds (7); the positive control with *MmCS* plasmid DNA (8), the negative control with water (9).

Wounding induction of *MmCS* expression

The induction of mRNA expression levels of *MmCS* in the basal leaves of *M. micrantha* through mechanical wounding at 5 days interval were determined with semi-quantitative RT-PCR analysis (Fig.4). The expression levels of *MmCS* in the leaves on the first day after wounding were significantly higher than those of control and other days. The expression levels of *MmCS* in the leaves sharply decreased on the second day. On the third day the expression levels were still significantly higher than in control (without wounding). Wounding and fungal elicitor induces the transcription of β-caryophyllene synthase in the basal leaves of *A. annua* within 24 h (3). A recent study showed that over-expressed rice sesquiterpene synthase gene, namely OsTPS3 ((*E*)-β-caryophyllene synthase), has led to more production of (*E*)-β-caryophyllene after MeJA treatment, and the MeJA-treated transgenic rice plants attracted more parasitoid wasps of *Anagrus nilaparvatae* than the wild-type (5). Similar to other volatile organic compounds, sesquiterpenes often accumulate in leaves, roots and rhizomes of many plant species (21). Sesquiterpenes are usually major plant volatile compounds induced by herbivore damage and they may play an important role in indirect defence of plants (22).

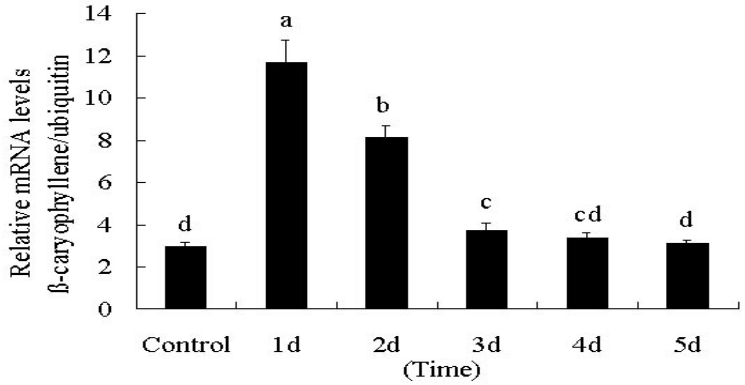

Figure 4. RT-PCR analysis of β-caryophyllene synthases transcripts in wounded leaves of *M. micrantha*.

Phytotoxicity of β-caryophyllene

Bioassay showed that β-caryophyllene at 0.375 mg L^{-1} stimulated the shoot length and root length of test plants (Table 1). However, the volatile at 3 mg L^{-1} concentration inhibited the seed germination, shoot and root growth of *B. campestris* by 17.0%, 15.5% and 11.6%, respectively. This concentration also inhibited the seed germination, shoot and root growth of *R. sativus* by 13.9%, 18.2% and 12.0%, respectively. Many allelochemicals have been demonstrated to have this hormesis phenomenon (1). Low concentration of β-caryophyllene also facilitated the growth of tested plants, but the high concentrations inhibited the growth (Fig.5). This suggested that volatile compounds also have hormesis phenomenon.

Table 1. Effects of β-caryophyllene on seed germination and seedling growth of *B. campestris* and *R. sativus*. All data were presented as means ± SD. Different letters in the same row indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

β-caryophyllene conc (mg L^{-1})	*B. campestris*			*R. sativus*		
	Germination (%)	Shoot height (mm)	Root length (mm)	Germination (%)	Shoot height (mm)	Root length (mm)
0 (Control)	88.3±7.6 a	11.6±4.4 b	43.6±9.7 b	93.3±2.9 a	22.9±6.3 b	58.2±17.1 b
0.375	98.3±2.9 a	13.2±3.6 a	49.8±9.6 a	95.0±5.0 a	27.5±6.6 a	64.9±12.0 a
3	73.3±7.6 b	9.8±4.1 c	38.0±14.4 c	80.0±5.0 b	18.8±7.2 c	51.7±13.1 c
24	70.0±5.0 b	8.5±4.2 c	28.9±11.9 d	76.7±10.4 b	16.6±5.9 c	39.3±8.6 d

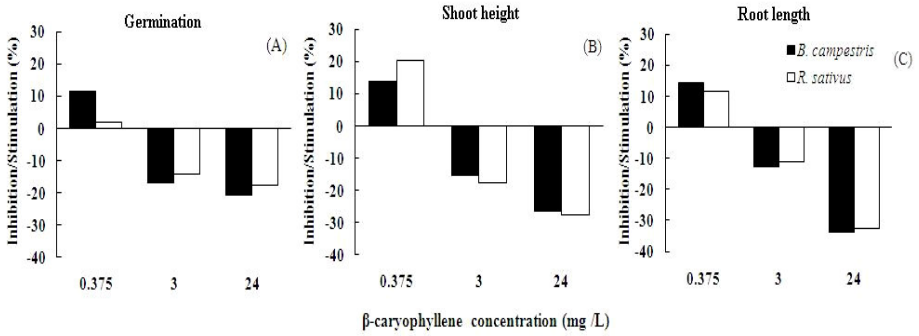

Figure 5. Effects of β-caryophyllene on germination (A), shoot height (B) and root length (C) of *B. campestris* and *R. sativus* plants. Data are expressed as RI values.

Feng *et al* (10) analyzed the volatile oil of *M. micrantha* and found 9.49% β-caryophyllene in the stems and leaves; Shao *et al* (23) found that volatile oil of its flowers contained 9.17% β-caryophyllene. Our this study also showed that β-caryophyllene was also a major sesquiterpene (9.72%) in *M. micrantha* leaves. Bioassay showed that this allelochemical inhibited the germination rate and seedling growth of *B. campestris* and *R. sativus*. Our result was consistent with previous studies on the allelopathic potential of this chemical (15,16).

This study cloned the β-caryophyllene synthase cDNA *MmCS* and further demonstrated that *MmCS* expression could be induced by mechanical damage. β-Caryophyllene, synthesized by the action of *MmCS*, had allelopathic potentials against test plants. These results indicated that β-caryophyllene may play an important role in alleopathy, as well as plant defence induced by herbivore damage in the invasive *M. micrantha* plants. Ismail and Chong (13) demonstrated that *M. micrantha* debris had allelopathic potential against tomato and Chinese cabbage. More work should be done to determine whether or not *M. micrantha* can accumulate enough concentrations of volatiles to interfere with growth of native plants in natural community in the future.

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