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MOLECULAR CLONING AND EXPRESSION LEVELS OF THE MONOTERPENE SYNTHASE GENE (*ZMM1*) IN CASSUMUNAR GINGER (*ZINGIBER MONTANUM* (KOENIG) LINK EX DIETR.)

SAOWALUCK BUA-IN[1], YINGYONG PAISOOKSANTIVATANA[1], BART C. WEIMER[2] and SRIMEK CHOWPONGPANG[3*]

[1] *Department of Horticulture, Faculty of Agriculture, Kasetsart University,* 10900, Bangkok, Thailand
[2] *Population Health & Reproduction, School of Veterinary Medicine,* University of California, Davis, USA
[3] *Department of Plant Pathology, Faculty of Agriculture,* Kasetsart University, Bangkok, Thailand

*Corresponding author: sbuain@gmail.com

Abstract - Cassumunar ginger *(Zingiber montanum* (Koenig) Link ex Dietr.) is a native Thai herb with a high content and large variety of terpenoids in its essential oil. Improving the essential oil content and quality of cassumunar ginger is difficult for a breeder due to its clonally propagated nature. In this research, we describe the isolation and expression level of the monoterpene synthase gene that controls the key step of essential oil synthesis in this plant and evaluate the mechanical wounding that may influence the transcription level of the monoterpene synthase gene. To isolate the gene, the selected clones from DNA derived from young leaves were sequenced and analyzed and the monoterpene synthase gene from cassumunar ginger (*ZMM1*) was identified. The *ZMM1* CDS containing 1 773 bp (KF500399) is predicted to encode a protein of 590 amino acids. The deduced amino acid sequence is 40-74% identical with known sequences of other angiosperm monoterpene synthases belonging to the isoprenoid biosynthesis C1 superfamily. A transcript of *ZMM1* was detected almost exclusively in the leaves and was related to leaf wounding. The results of this research offer insight into the control of monoterpene synthesis in this plant. This finding can be applied to breeding programs or crop management of cassumunar ginger for better yield and quality of essential oil.

Key words: gene isolation; terpene synthase; transcript; leaf; terpinen-4-ol.

INTRODUCTION

Monoterpenes are the simplest class of terpenoid in that they contain only 10 carbon atoms of terpenoids, commonly present in plant essential oils. These can be extracted from plants and are industrially valuable and utilized for commercial purposes, such as fragrances and anticancer pharmaceuticals and agrochemicals for aphid pheromones. Monoterpenes are derived from isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). In plants, this central precursor is synthesized in the plastids via the alternative pyruvate/2-methyl-D-erythritol-4-phosphate (MEP) pathway. The plastidial pathway also provides precursors for the biosynthesis of isoprene (C5), diterpenes (C20), and tetraterpenes (C40) (Mahmoud and Croteau, 2001). The regulation of plant terpenoid biosynthesis is complicated in large part because of the manifold functions of this family of metabolites and the vast differences in control in both time and location throughout the course of plant development.

In general, it contains two broad categories, spatial and temporal control or short periods of transient defense reaction from biotic and abiotic factors, such as insect or pathogen damage, light intensity, temperature, humidity and nutrient availability (Cheng et al., 2007; Yu and Utsumi, 2009)

Terpene synthases (TPSs) are the primary enzymes responsible for catalyzing the formation of essential terpene synthesis. Significant achievements have been made in metabolic engineering to increase terpenoid production (Cheng et al., 2007). Hundreds of terpene synthases have been characterized to date and knowledge of the structural and mechanistic properties of these enzymes has increased tremendously (Aubourg et al., 2002; Dudareva et al., 2003; Tholl, 2006a). Amino acid sequence relatedness of plant terpene synthases allows subdivision of the *Tps* gene family into six subfamilies, designated *Tps-a* through *Tps-f*, distinguished from each other by sharing a minimum of 40% identity among members. However, all plant terpene synthase share a common evolutionary origin (Bohlmann et al., 1998) and one characteristic feature of most terpene synthases is the formation of multiple products from a single substrate.

Monoterpene synthases (cyclases) are the key enzymes in monoterpene biosynthesis (Croteau, 1987), as they catalyze the cyclization of the ubiquitous geranyl diphosphate (GPP/GDP), the universal acyclic C10 intermediate of isoprenoid biosynthesis, to the specific monoterpene skeletons (El Tamer et al., 2003; Schwab et al., 2001). Functional analysis of the monoterpene synthases suggests that each monoterpene synthase produces specific main products, whereas several monoterpene synthases are able to produce more than one product (McGarvey and Croteau, 1995). This shows that monoterpene synthases may have unique mechanisms to control the specific deprotonation or cyclization of carbocations to produce a wide range of monoterpenoids. In recent years, severa1 genes involved in terpenoid biosynthesis have been cloned and characterized from various gymnosperm and angiosperm species, including the genes for monoterpene synthases, ie., sage (*Salvia officinalis*) (Wise et al., 1998), conifers (*Abies grandis* and *Taxus brevifolia*) (Trapp and Croteau, 2001), snapdragon (*Antirrhinum majus*) (Dudareva et al., 2003), grape (*Vitis vinifera*) (Martin and Bohlmann, 2004), tea tree (*Melaleuca alternifolia*) (Shelton et al., 2004) and Satsuma mandarin (*Citrus unshiu* Marc.) (Shimada et al., 2005; Shimada et al., 2004). However, there is no report on monoterpene synthase gene isolation in *Zingiber* species.

Cassumunar ginger (*Zingiber montanum* (Koenig) Link ex Dietr.) belongs to the Zingiberaceae family. It occurs widely as a home-garden plant in Southeast Asia. Recently, cassumunar ginger has been identified as an important but underutilized medicinal crop. The rhizomes' essential oil can relieve joint and as a muscle-pain. Being in the ginger family, it gives a cooling instead of warming effect on inflamed areas. This plant also has antioxidant activity, antiseptic, antitoxic, antiviral, digestive and strong anti-inflammatory properties (Jeenapongsa et al., 2003; Poonsukcharoen, 2004). Terpene hydrocarbon is the main constituent of cassumunar ginger essential oil. Most are monoterpenes, presented as acyclic, monocyclic or bicyclic, such as sabinene (33.99-47.54%) and terpinen-4-ol (11.5-24.36%) (Bua-in and Paisooksantivatana, 2009; Manochai et al., 2007). Of the compounds found in cassumunar ginger, only terpinen-4-ol and (*E*)-1(3′, 4′-dimethylphenyl) butadiene (DMPBD) have confirmed anti-inflammatory activity (Jeenapongsa et al., 2003; Poonsukcharoen, 2004).

Due to the popularity of cassumunar ginger as a medicinal plant for pain relief, there is a rapidly increasing demand for raw material for drug development. In the pharmaceutical and cosmetic industries, the main value of cassumunar ginger is linked to the amount of terpinen-4-ol in the rhizome oil. For this reason, the Thailand Institute of Scientific and Technological Research (TISTR) established the cassumunar ginger oil standard with a minimum terpinen-4-ol content of 36% and less than 5% of DMPBD. However, major problems in drug development from cassumunar ginger are the uneven quality and quantity of the raw material. There is little room for improv-

ing the essential oil content and quality of this plant through breeding because it is a clonally propagated species; the best opportunity for improving yield and quality is through environmental manipulation. In addition, the mechanisms of essential oil accumulation regulation at the physiological, biochemical and genetic levels are still unclear. Moreover, the biosynthesis of the main constituent terpinen-4ol is under discussion. Higher contents of terpinen-4-ol in the essential oil of plants, such as marjoram and tea tree, are derived by rearrangement from another oxygenated monoterpene, sabinene hydrate, during the distillation process (Fischer et al., 1988; Lawrence, 1997; Shelton et al., 2004). Therefore, it is necessary to identify and characterize the genes involved in the production essential oil constituents in cassumunar ginger and their expression under particular stress environments. This research investigated the cloning and expression of monoterpene synthase genes controlling the major step of essential oil constituents' synthesis in cassumunar ginger.

MATERIALS AND METHODS

Plant materials

Zingiber montanum from Rachaburi province, Thailand was grown under tissue culture conditions. The rhizomes' buds were cultured in MS medium (Murashige and Skoog, Basal Medium, w/vitamin *Phyto*Technology Laboratories, LLC™, KS) with the supplement of sucrose 1.6% w/v, BAP 1mg/l, NAA 0.1 mg/l, ferrous sulfate 6.67 mM and 5% w/v of activated charcoal, pH 5.7. Cultures were incubated in an A1000 (Conviron, Manitoba, Canada) growth chamber at a constant temperature of 26±1°C, under 16 h light/day at 200 μmoles/m^2/s.

Genomic DNA extraction, oligo nucleotide primers design and monoterpene synthase gene cloning.

Young leaves were cut, frozen and ground in liquid nitrogen using mortar and pestle. Genomic DNA was extracted with a DNeasy Plant Mini Kit (Qiagen). The monoterpene synthase gene was amplified from genomic DNA by conventional PCR. Two primers, 5'-GATGATATTTACGATGTCTATGGA-3' and 5'-ATATTTTGGCACGTCGCCTCT-3' were designed by reverse translation of the amino acid sequences of monoterpene synthases isolated from plants that revealed highly conserved boxes (http://www.ncbi.nlm.nih.gov/) compared to ginger-EST from the Aromatic Rhizome EST database (ArREST) of the University of Arizona (http://ag.arizona.edu/research/ganglab/ArREST.htm). The PCR protocol was used as described by GoTaq® DNA Polymerase Kit (Promega) in the Bio-Rad CFX96™ real-time PCR (Bio-Rad Laboratories, Inc.). The annealing temperature was 51.5°C and the extension time was 1 min. The 750 bp PCR product was purified and cloned into the RBC TA Cloning Vector Kit (RBC Bioscience Corp., New Taipei City 23145, Taiwan) and transformed into *E. coli* DH5α competent cells, and DNA sequenced. The partial sequence was extended toward 5' end and 3' end by inverse PCR (IPCR) method. The ten restriction enzymes used for inverse PCR were *BamH*I, *EcoR*I, *Pci*I, *Sph*I, *Xba*I, *Pst*I, *Sal*I, *Cla*I, *Xma*I and *Mfe*I and T4 DNA ligase was used for catalyzed the circularization of the fragment DNA. Each IPCR was amplified by using oligonucleotide primers (Table 1) that were designed from the first fragment cloned above. PCR conditions were as follows: 94°C for 5 min, 35 cycles of three steps (94°C for 1 min, 50°C for 45 s and 72°C for 2 min) and extended with 10 min at 72°C. The PCR products were cloned into the RBC TA Cloning Vector Kit, analyzed to determine the nucleotide sequence of the open reading frame and confirmed by sequence. Intron junctions were mapped and spliced by using FGENESH (available in website http://linux1.softberry.com). *ZMM1* was confirmed by conventional PCR using two primers, ZMMF 5'- ATGTCTCTCTTCCACCCAGCA-3' and ZMMR 5'-CTAAATTTGGATAGG TTCGAGGAAC-3'.

Sequence analysis

A similarity search of the *ZMM1* sequence in public nucleotide and protein databases was performed using blastn and blastp algorithms, respectively (available at the National Center for Biotechnology Information (NCBI) web site (http://blast.ncbi.nlm.nih.gov/

Blast.cgi)) (Altschul et al., 1997). Multiple alignment and phylogenetic analysis of the plant monoterpene synthases were carried out using ClustalW2 (Larkin et al., 2007) for comparison of the deduced amino acid sequence of *ZMM1* with those of the known monoterpene synthases as follows: NP_001268216 (*Vitis vinifera*: (-)-α-terpineol synthase), ADR74206 (*Vitis vinifera*: (E)-β-ocimene/myrcene synthase), ADX01381 (*Citrus hystrix*: pinene synthase), XP_004161822 (*Cucumis sativus*: (-)-α-terpineol synthase-like), AEJ91556 (*Litsea cubeba*: α-thujene synthase/sabinene synthase), NP_001233805 (*Solanum lycopersicum*: monoterpene synthase 1), AER12203 (*Hedychium coccineum*: chloroplast linalool synthase) and AB247331 (*Zingiber zerumbet*: α-humulene synthase). The phylogenetic tree was constructed using amino acid sequence alignment by clustal method using the TREECON program for Windows (Van de Peer and De Wachter, 1994).

Gene expression analysis among different tissues

Total RNA for RT-PCR and real-time qPCR from old rhizomes, young rhizomes, pseudostems and leaves from the pot plants was isolated with NucleoSpin® RNA Plant (MACHEREY-NAGEL GmbH & Co. KG) and was quantified by using a spectrophotometer. Complementary DNA (cDNA) was synthesized from 500 ng of the total RNA by reverse transcription (RT) reaction using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., MA, USA) with attached oligo-$(dT)_{18}$ primer according to the manufacturer's instruction. Transcription of *ZMM1* in each tissue was monitored by RT-PCR, a fragment of 135 bp was amplified with the specific primers: forward (sense) 5'-GCCAACGACTTAACTGGAGAGGCCTTGA-3' and reverse (antisense) 5'- TGGCACGTCGCCTCTTTCTAGCTCAT-3'. *Ubiquitin* endogenous control primers were forward 5'-AAGGAGTGCCCCAACGCCGAGTG-3' and reverse 5'-GCCTTCTGGTTGTAGACGTAGGTGAG-3' (Yu et al., 2008). PCR for *ZMM1* and *ubiquitin* were performed for 35 cycles with an annealing temperature of 60°C. The relative quantification was carried out for 35 cycles by a SensiFast SYBR No-Rox kit (Bioline Ltd, UK). The relative expression ratios were quantified by the $\Delta\Delta C_t$ method (Ramakers et al., 2003).

Gene expression analysis in response to leaf wounding

Zingiber montanum was grown in tissue culture under the same conditions as for gene cloning. Wounding was performed on 90- and 60-day-old explants. Fully expanded leaves were crushed once across the apical laminar using serrate forceps so that approximately 10-20% of the total leaf area was wounded. Explants were incubated under the light for 15 (T15) and 45 (T45) min after wounding; then the leaves were harvested and immediately frozen in liquid nitrogen. Leaves harvested immediately after wounding were the positive control (T0) and the unwounded leaves served as control. Samples were then kept in a -80°C freezer until extraction. Three independent biological replicates of *Z. montanum* leaves were used. Total RNA from the wounded leaves was extracted. The RT-PCR and real-time qPCR for five of each sample and endogenous control were amplified in separate wells following the same procedure as that of gene expression analysis among different tissues.

RESULTS AND DISCUSSION

Isolation of a novel terpene synthase gene, ZMM1, from cassumunar ginger

Conventional PCR was performed with DNA extracted from the young leaves of *Z. montanum* with specific primers based on amino acid sequences of monoterpene synthase from divergent angiosperm species and the Aromatic Rhizome EST database (ArREST) of *Z. officinale*. In this study, we preferred to use genomic DNA instead of total RNA, because the yield of RNA from the aromatic plant is low and its quality is poor. However, there are some reports showing the development of protocols for rapid and simplified extraction of total RNA from plants containing high levels of starch and phenolics (Appalasamy et al., 2012; Kumar et al., 2007).

A 734 bp fragment showing homology to plant monoterpene synthase was amplified. The 5' and 3'

Table 1. List of primers using in inverse PCR method.

Name	Sequence 5-3	TM
ZMIPCRR1	TCC ATA GAC ATC GTA AAT ATC ATC	50.6
ZMIPCRR2	T TGT CCA TGG CAG CTA AGT CCC	56.7
ZMIPCRR3	TTC ATG TAT TCT GGA AGC TTG TC	51.7
ZMIPCRR4	CAA GCC CTT CTC CTT CAT CAC	54.4
ZMIPCRF1	AGA GGC GAC GTG CCA AAA TAT	52.4
ZMIPCRF2	GAT GAT ATG GGC ACT TCC ACG	54.4
ZMIPCRF3	GAG ATC ACA CGC TCC TCT AG	53.8
ZMIPCRF4	GAT TGG TAT CAA TAG CTG GTC C	53

ends of the coding region were obtained by inverse PCR. Inverse PCR extends the 5' and 3' ends on the sense strand (Digeon et al., 1999). The restriction enzyme *SphI* was used. The translated protein was 590 amino acids. Results from intron splicing of 1 773 bp, *ZMM1* revealed seven exons and six introns, which included the poly A at C-terminal. Therefore, *ZMM1* belongs to the isoprenoids biosynthesis C1 superfamily or Class-III TPS gene subfamily, which consist of seven conserved exons and is interrupted by six introns and represents the only angiosperm monoterpene, sesquiterpene and diterpene synthases (Aubourg et al., 2002; Bohlmann et al., 1998).

Amino acid sequence analysis of *Zingiber montanum* monoterpene synthase (ZMM1) (http://www.ncbi.nlm.nih.gov/genbank/) indicated that the encoded protein is 40 to 74% identical with known sequences of other angiosperm monoterpenes (Fig. 1). The most significant similarity (74% identity) was to the chloroplast linalool synthase (accession no. AER12203) (Li and Fan, 2011) in butterfly ginger (*Hedychium coccineum* Buch.-Ham. ex Sm.). In similarity comparison to dicotyledon species, the ZMM1 showed 50% identity similar to linalool synthase (-)-α-terpineol synthase in *Vitis vinifera*: accession no. NP_001268216) (Martin and Bohlmann, 2004). There is a lack of reports on the *Zingiber* monoterpene synthase gene (Fujisawa et al., 2010; Picaud et al., 2006; Yu et al., 2008). Thus, *ZMM1* was the first monoterpene synthase gene reported in this group of plant. This finding suggested that the *ZMM1* fragment originated from the same ancestor as other terpene synthase genes in angiosperm, supporting the view that plant terpene synthase share a common evolutionary origin (Back and Chappell, 1995; Bohlmann et al., 1998). One characteristic feature of most terpene synthases is the formation of multiple products from a single substrate.

The ZMM1 contained the aspartate-rich DDxxD-motif (Tholl, 2006b), and the RR(x)8W-motif, which are conserved among plant terpene synthases (Aharoni et al., 2004; Dudareva et al., 2003; Lücker et al., 2002; Tholl, 2006a) and play an important role in monoterpene synthesis. They are supposed to be the putative Mg^{2+} and Mn^{2+} binding site (Aros et al., 2012; Williams et al., 1998) during monoterpene isomerization or cyclization (Roeder et al., 2007). In addition, ZMM1 contained three more terpene synthase conserved sequence motifs: the RWW-motif, RxR-motif and CYMNE-motif (Fähnrich et al., 2011; McKay et al., 2003) (Fig. 1).

Fig. 1. Amino acid sequence alignment of the monoterpene synthases. Motif consensus amino acids are shaded. The dendrogram includes *Vitis vinifera* (-)-α-terpineol synthase (NP_001268216), *Vitis vinifera*: (E)-β-ocimene/myrcene synthase (ADR74206), *Citrus hystrix*: pinene synthase (ADX01381), *Cucumis sativus*: (-)-α-terpineol synthase-like (XP_004161822), *Litsea cubeba*: α-thujene synthase/sabinene synthase (AEJ91556), *Solanum lycopersicum*: monoterpene synthase 1 (NP_001233805), *Hedychium coccineum*: chloroplast linalool synthase (AER12203) and ZMM1. Identity was calculated after ClustalW alignment using JalView Pairwise alignment tool.

Phylogenetic analysis of ZMM1

Base on the amino acid sequence, a phylogenetic tree (Fig. 2) clearly showed that the ZMM1 was grouped together with angiosperm terpene synthase (TPSs). As a result, ZMM1 was placed with the monoterpene synthase in other dicotyledons rather than with the sesquiterpene synthase in monocotyledons within the same genus. Linalool synthase from *Hedychium coccineum* (JN695016.1: 74% identity) is closest to

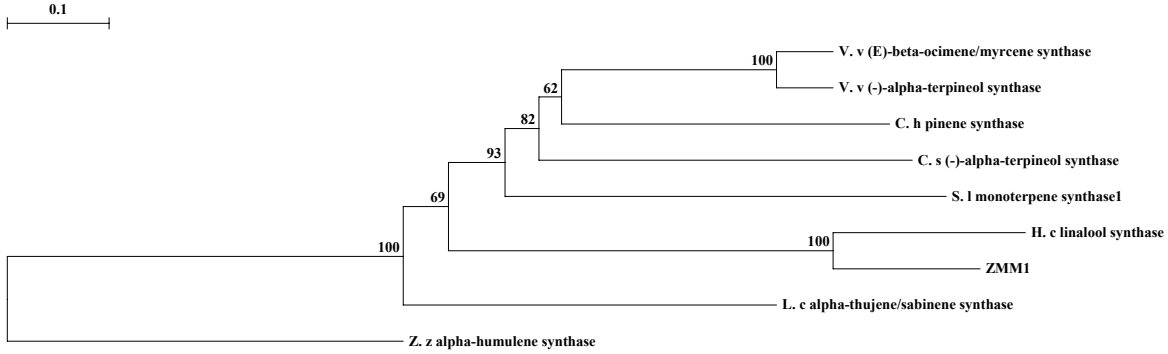

Fig. 2. Phylogenetic tree of plant monoterpene synthases. The dendrogram includes *Vitis vinifera*: (-)-α-terpineol synthase (NP_001268216), *Vitis vinifera*: (E)-β-ocimene/myrcene synthase (ADR74206), *Citrus hystrix*: pinene synthase (ADX01381), *Cucumis sativus*: (-)-α-terpineol synthase-like (XP_004161822), *Litsea cubeba*: α-thujene synthase/sabinene synthase (AEJ91556), *Solanum lycopersicum*: monoterpene synthase 1 (NP_001233805), *Hedychium coccineum*: chloroplast linalool synthase (AER12203), *Zingiber zerumbet*: α-humulene synthase (AB247331) and ZMM1. The bootstrap values are representing at the start of the branch. The scale bar represents the distance estimation by the Jukes and Cantor model.

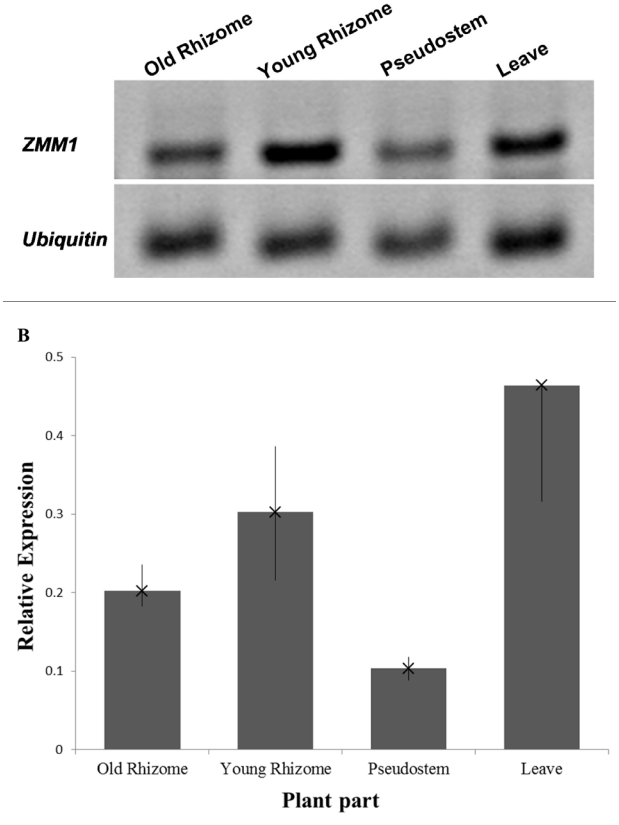

Fig. 3. Tissue-specific expression of *ZMM1*. Total RNA was isolated from olde rhizomes, young rhizomes, pseudostems and leaves: (A) 1% (m/v) agarose gel showing the 235bp product by RT-PCR. (B) Represent real time-PCR relative expression analysis. Results represent mean ± SEM of 3 independent experiments. *Ubiquitin* served as an endogenous control.

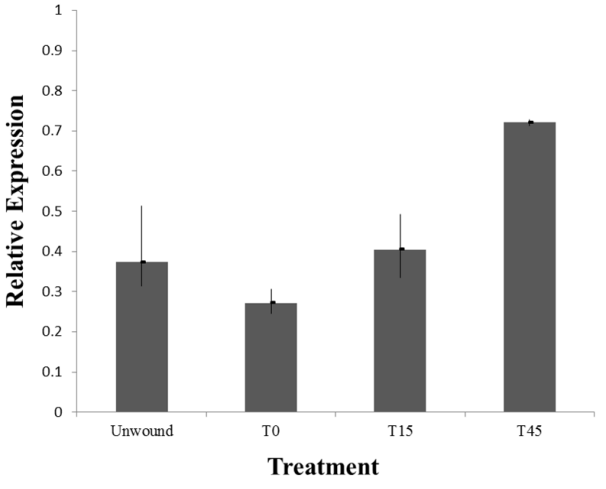

Fig. 4. The relative expression of *ZMM1* in response to mechanical wounding. Total RNA was isolated from the leaves of 60-d-old plant which crushed once across the apical laminar and collected at 0 (T0), 15 (T15) and 45 min (T45) after wound infliction. Results represent mean ± SEM of 5 independent experiments. *Ubiquitin* served as an endogenous control.

ZMM1, while the *Zingiber zerumbet* sesquiterpene synthases is independent from other monoterpene synthase genes. The sesquiterpene synthase from *Zingiber officinale* (accession no. AB511914 and BAI67935) and *Zingiber zerumbet* (accession no. AB247331 and AB247334) showed 32%, 32%, 32% and 32% identity to ZMM1, respectively. The phylo-

genetic trees of most terpene synthases indicates the formation of multiple products from a single substrate (Aubourg et al., 2002). In addition, the phylogenetic comparison confirmed that, within the same plant genome, TPSs produce different products that may be more similar to each other than TPSs that produce the same products in different species, suggesting that there were a convergent evolution in this family of proteins (Bohlmann et al., 1998; Chen et al., 2004; Lin et al., 2008).

Gene expression analysis among different tissues

To determine the expression levels of *ZMM1,* mRNAs in various tissues of *Z. montanum*, were examined by RT-PCR analysis with total RNA isolated from leaves, pseudostems, 1-year-old rhizomes and newly formed rhizomes. The *ZMM1*-specific primers were designed from the two introns of the *ZMM1* genomic sequence. The partial cDNA sequence of an *Ubiquitin* was used as an androgynous standard reference. The *ZMM1* transcript was detected in young rhizomes, whereas its levels were lower in leaves, old rhizomes and pseudostems (Fig. 3A). We also performed quantitative PCR to compare the transcription levels of *ZMM* (Fig. 3B). Our results show that the transcription level in the leaves was the same, two- and three-fold higher in young rhizomes, old rhizomes and pseudostems, respectively. Bhuiyan et al. (2008) reported that the contents of monoterpenes such as terpinen-4-ol and ocimene in the essential oil from rhizomes was higher than in the leaves of *Z. montanum*, whereas the contents of monoterpenes such as sabinene, β-pinene and caryophyllene contents were higher than in the rhizome. Therefore, ZMM1 end-products might be monoterpenes that are present in the leaves more than in the rhizomes. However, our study was performed using pot-grown plants in green house, and these results need to be confirmed in plants growing in the wild.

Gene expression analysis in response of leave wounding

To examine the inducible expression pattern in the leaf, we used quantitative PCR patterns to compare the transcription levels of *ZMM1* in wounded leaves as compared to unwounded control leaves, over 45 min. Mechanically wounding is an abiotic factor which induced the activation of many different genes, and can be contributed to tissue defense and repair mechanisms in higher plants, such as *Arabidopsis* (Reymond et al., 2000), Sitka spruce (Byun-McKay et al., 2006) and grand fir (Steele et al., 1998). In this study, transcription in wounded leaves which were harvested immediately after wound infliction was downregulated, and upregulated two-fold at 45 min post-wounding (Fig. 4). This result suggests that mechanical wounding immediately suppressed the expression level of *ZMM1* in leaves which subsequently increased (Zhou and Thornburg, 1999). The result is in agreement with the finding obtained from work on rice terpene synthase genes that suggests that plant metabolites, especially terpenoids, are most often regulated at the level of transcription (Wilderman et al., 2004). The expressions of several terpene synthase genes are often stimulated by treatment with MeJA, or by mechanical or insect damage (Gomez et al., 2005; Yu et al., 2008). Mechanical wounding could provide valuable tools for further improving yield of important terpenoids in cassumunar ginger.

In this study, we provide the first identidfication of the monoterpene synthase gene from cassumunar ginger (*ZMM1*), which has been deposited into GenBank with accession number KF500399. Phylogenetic analysis clearly shows that ZMM1 can be grouped together with other terpene synthase genes from angiosperms. The transcript of *ZMM1* was detected almost exclusively in the leaves. Our current interpretation of the result is that *ZMM1* might control the biosynthesis of some monoterpenes such as sabinene and β-pinene in the leaves.

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