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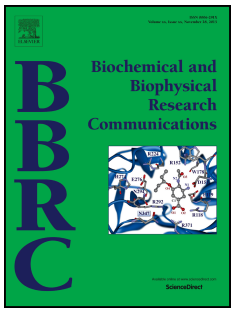

Functional characterization and transient expression manipulation of a new sesquiterpene synthase involved in β-caryophyllene accumulation in *Ocimum*

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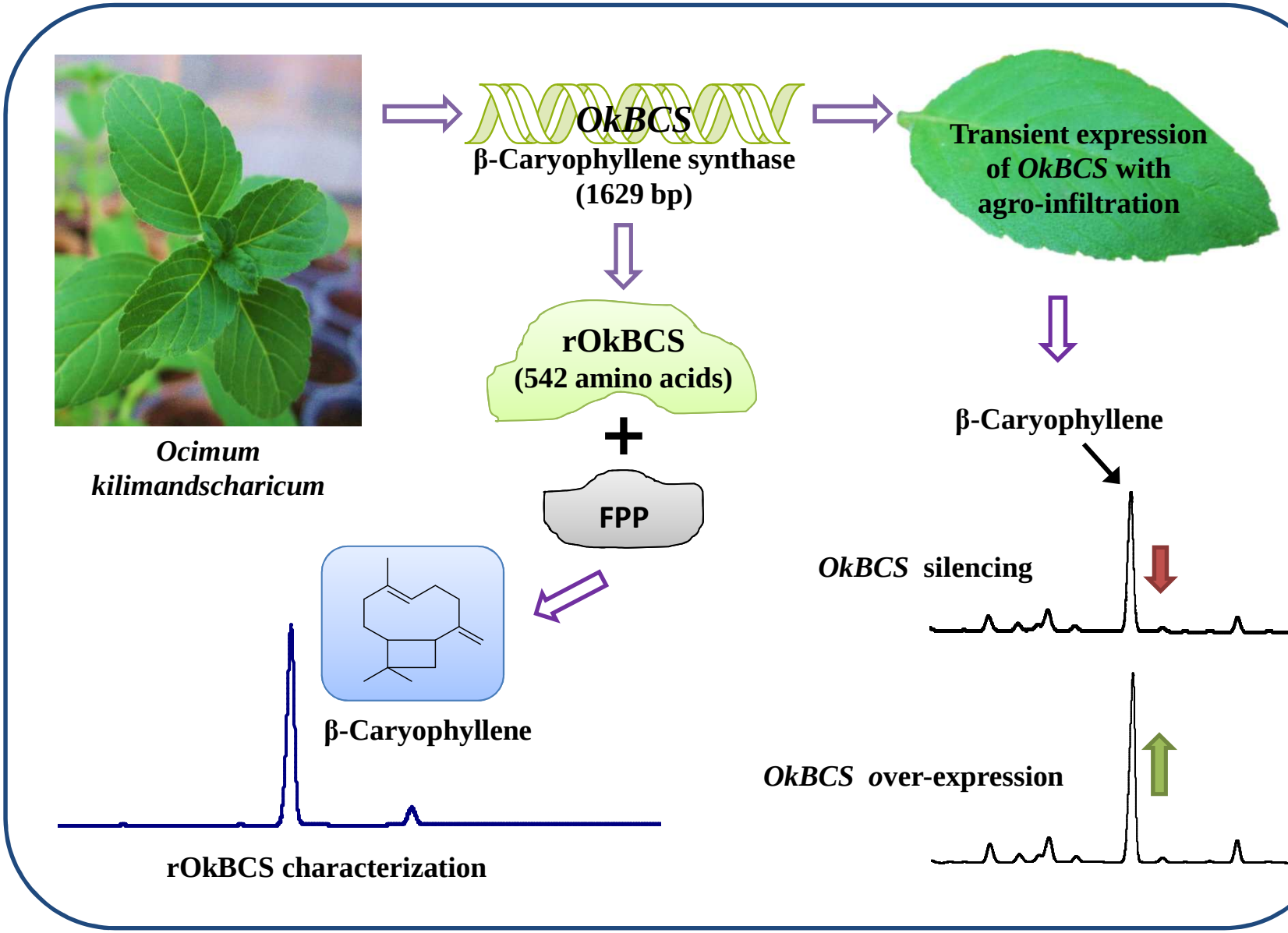
OkBCS
β-Caryophyllene synthase
(1629 bp)
Transient expression
of OkBCS with
agro-infiltration
Ocimum
kilimandscharicum
rOkBCS
(542 amino acids)
+
FPP
β-Caryophyllene
β-Caryophyllene
OkBCS silencing
OkBCS over-expression
rOkBCS characterization

Functional characterization and transient expression manipulation of a new sesquiterpene synthase involved in β-caryophyllene accumulation in *Ocimum*

Ramesha H. Jayaramaiah[1#], Atul Anand[1, 2#], Supriya D. Beedkar[1, 3], Bhushan B. Dholakia[1], Sachin A. Punekar[4], Raviraj M. Kalunke[1], Wasudeo N. Gade[3], Hirekodathakallu V. Thulasiram[2,5,*], Ashok P. Giri[1,*]

[1]Division of Biochemical Sciences and [2]Division of Organic Chemistry, CSIR-National Chemical Laboratory, Pune 411008, Maharashtra, India

[3]Department of Biotechnology, Savitribai Phule Pune University, Pune 411007, Maharashtra, India

[4]Biospheres, Eshwari, 52/403, Lakshminagar, Parvati, Pune 411 009, Maharashtra, India

[5]CSIR- Institute of Genomics and Integrative Biology, Mall Road, New Delhi 110007, India

[#] Equal contribution

*****Corresponding authors:

H. V. Thulasiram; Tel.: +91 2025902478; Fax: +91 2025902624; hv.thulasiram@ncl.res.in

Ashok P. Giri; Tel.: +91-20-25902710; Fax: +91-20-25902648; ap.giri@ncl.res.in

Abstract

The genus *Ocimum* has a unique blend of diverse secondary metabolites, with major proportion of terpenoids including mono- and sesqui-terpenes. Although, β-Caryophyllene, bicyclic sesqui-terpene, is one of the major terpene found in *Ocimum* species and known to possess several biological activities, not much is known about its biosynthesis in *Ocimum*. Here, we describe isolation and characterization of β-caryophyllene synthase gene from *Ocimum kilimandscharicum* Gürke (*OkBCS*- GenBank accession no. KP226502). The open reading frame of 1,629 bp encoded a protein of 542 amino acids with molecular mass of 63.6kDa and pI value of 5.66. The deduced amino acid sequence revealed 50-70% similarity with known sesqui-terpene synthases from angiosperms. Recombinant OkBCS converted farnesyl diphosphate to β-caryophyllene as a major product (94%) and 6% α-humulene. Expression variation of *OkBCS* well corroborated with β-caryophyllene levels in different tissues from five *Ocimum* species. *OkBCS* transcript revealed higher expression in leaves and flowers. Further, agro-infiltration based transient expression manipulation with *OkBCS* over-expression and silencing confirmed its role in β-caryophyllene biosynthesis. These findings may potentially be further utilized to improve plant defense against insect pests.

Keywords: β-caryophyllene; Terpene synthase; Transient expression; *Ocimum*; Natural product biosynthesis

1. Introduction

Terpenes are the largest group of natural products, present ubiquitously with immense diversity in their structure and function [1]. They perform versatile functions including communication and defense in plants [2]. Several terpenes that offer distinct fragrance to flower are crucial in attracting many insects and other pollinators [3]. Plants use different defense strategies for their survival and one of them is to recruit natural enemies of herbivores using induced volatiles. Among herbivore-induced volatiles, terpenoids that comprise mono- and sesqui-terpenes, are the most commonly utilized compounds as tactical arsenal [4]. Like other members of the Lamiaceae family, *Ocimum* plant synthesizes and accumulates these volatile compounds in the secretory capitate and peltate trichomes which are located on the surface of aerial parts of plants [5]. Mono- and sesqui-terpenes are the main constituents of volatile components, which impart them unlimited medicinal properties [6] as well as a characteristic flavor and taste of *Ocimum* [7]. Several species from the genus *Ocimum* are known to possess insecticidal and other important bioactive properties [8,9].

β-caryophyllene, a bicyclic sesqui-terpene, is widely distributed in plant kingdom including *Ocimum* species [10]. Like most terpenes, it contributes unique aroma in essential oils from numerous plants. β-caryophyllene might be effective for treating cancer, anxiety and depression [11,12] and FDA has approved it as food additive. Additionally due to insecticidal nature, it could be potentially useful in plant defense [9,13]. Though terpenoid biosynthesis from genus *Ocimum* has received considerable attention, very little is known on β-caryophyllene biosynthesis in *Ocimum.* Here, we describe molecular characterization of β-caryophyllene synthase from *Ocimum kilimandscharicum* Gürke (OkBCS). Correlation of *OkBCS* transcript

levels with metabolic variations in different tissues from five *Ocimum* species was performed. Further, transient manipulation of *OkBCS* expression in terms of silencing and over expression validated its role in β-caryophyllene biosynthesis.

2. Materials and methods

2.1. Chemicals and plant materials

All chemicals were purchased from Sigma-Aldrich (Sigma Chemical Co., USA), unless stated. Authenticated *Ocimum* plants were grown in a greenhouse at 24ºC, humidity 35-40%, 16h light and 8h dark period. Voucher specimens of all the species were deposited in the herbarium of Botanical Survey of India, Pune. Different tissues were snap frozen in liquid nitrogen and stored at -80ºC until further use.

2.2. Isolation of β-caryophyllene synthase (BCS) from Ocimum kilimandscharicum

Putative *Ocimum* terpene synthase (TPS) encoding genes were identified using Blastx by comparing known *TPS* with de novo assembled *Ocimum* transcriptome (unreported). Coding sequence of *OkBCS* was obtained from *Ocimum kilimandscharicum* cDNA using full-length open reading frame (ORF) primers (Table S1). Total RNA was isolated from leaves using Spectrum Plant Total RNA Kit (Sigma-Aldrich, USA) and cDNA was synthesized using SuperScript™III reverse transcriptasekit (Invitrogen, USA). Full-length ORF of cDNA was cloned in Zero blunt vector (Invitrogen) and sequenced.

2.3. Heterologous expression and purification

For directional cloning into pET102 expression vector (Invitrogen), 5'-CACC-3' was added to the 5′ end of the forward primer and stop codons were removed in reverse primer to allow for C-terminal His-tag expression. The complete *OkBCS* ORF was amplified with AccuPrime™ Pfx DNA Polymerase (Invitrogen) and ligated into the pET102 vector producing pET102-*OkBCS*, in which the *OkBCS* coding sequence was fused with a 6X His-tag-coding extension at the C-terminus and transformed into *Escherichia coli* TOP10 cells. The construct was verified by DNA sequencing. The sequence verified plasmids were introduced into the Rosetta 2 (DE3) cells. Expression and purification of recombinant protein was carried out as described earlier [14].

2.4. Biochemical characterization of recombinant OkBCS

Enzyme assays were performed in 10 mL glass tubes, using 10 µg of purified recombinant protein, 50 µM substrate (GPP or FPP), 10 mM $MgCl_2$ and 1 mM DTT in assay buffer to a total volume of 250 µL. The reactions were incubated for 30 min at 30°C. Later, assay mixture was extracted twice with 2 mL of n-Hexane. The combined organic phase was dried over anhydrous sodium sulphate and concentrated to 50 µL by flushing it with nitrogen. Enzymatic products were analyzed by GC-MS for identification of volatiles. *E. coli* cells transformed with empty vector and assays without substrates were used as controls. Steady state kinetics of recombinant OkBCS (rOkBCS) was performed in assay buffer containing 10 µM purified protein and substrate concentrations varying from 10-600 µM. The product amount and ratio were calculated using GC and used for the determination of K_m and K_{cat} values using GraphPad Prism ver. 6 (http://www.graphpad.com/scientific-software/prism/).

2.5. Sequence analysis

Sequence analyses were carried out with BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Nucleotide sequences were translated using ExPASy (http://web.expasy.org/translate/) and BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) was used for marking identical and similar amino acids. Multiple sequence alignment was performed using ClustalX 2.1 (http://www.clustal.org/clustal2/) and neighbour-joining (NJ) tree of 33 TPSs was reconstructed with MEGA 6 [15] with 1000 iterations.

2.6. Volatile extraction and GC-MS analysis

The volatiles β-caryophyllene and α-humulene were measured in six different tissues including young leaves, mature leaves, inflorescence, flower, stem and root from ten different individuals of five *Ocimum* species. Fresh tissues (5 g each) from 2-month-old plants were harvested separately in triplicates and immediately used for volatile extraction using GC-MS as described earlier [9].

2.7. OkBCS expression analysis

For quantitative RT-PCR (qRT-PCR), 1µg of total RNA was used for cDNA synthesis using High capacity cDNA reverse transcription kit (Applied Biosystems, USA). qRT-PCR was performed on an Applied Biosystems 7500 HT Fast Real-Time PCR. A typical 10 µL reaction consisted of 5 µL of SYBR green master-mix, 0.2 µL of 10µM forward and reverse gene specific primers (Table S1) and 1 µL of diluted cDNA (1:2) with nuclease-free water added to make up a volume of 10 µL. A relative quantification of gene expression was performed using elongation

factor-1α (EF-1α) as a reference gene. The difference in relative expression levels were calculated from the $2^{-\Delta\Delta Ct}$ value after normalization of *OkBCS* data to EF-1α. All analyses were performed in triplicates using five biological replicates for each sample.

2.8. Agrobacterium mediated transient over-expression and silencing of OkBCS

Full-length *OkBCS* was cloned into the pRI 101-AN vector for over-expression in plants, using *Sal*I and *Eco*RI restriction sites in forward and reverse primers, respectively (Table S1). A 826 bp region from ORF was used as sense fragment and its complementary sequence as anti-sense fragment. A 500 bp fragment from wheat starch branching enzyme was used as intron. Silencing construct was prepared by cloning sense, intron and anti-sense fragments in tandem in pRI 101-AN vector. Sense fragment was cloned using *Sal*I and *Kpn*I restriction sites, transformed in TOP10 cells (Invitrogen) and positive clones were verified by restriction digestion and sequencing. Similarly, intron was cloned using *Kpn*I and *Bam*HI restriction sites and anti-sense fragment was cloned using *Bam*HI and *Sac*I restriction sites. After sequence verification, it was transformed into *Agrobacterium tumefaciens* (GV3101) chemically competent cells on Luria-agar plate containing 50µg/mL kanamycin and 25µg/mL rifampcin. *A. tumefaciens* cells carrying over-expression or silencing construct were grown in 2 mL LB media containing these antibiotics at 28°C on rotary shaker set at 180 rpm for 2 days. This starter culture was used to inoculate 10 mL LB with the antibiotics and incubated over-night at 28°C and 180 rpm. Later, cells were pelleted down by centrifugation at 10,000 rpm for 10 mins at 4°C. Cells were washed with half strength MS media, pH 5.4 and pelleted again. This cell pellet was re-suspended in half strength MS media, pH 5.4 to bring it to an $O.D._{600}$ of 0.3-0.5. This suspension was used for syringe-assisted infection on the adaxial surface of the leaves. Five *Ocimum kilimandscharicum* plants of each were taken for *OkBCS* over-expression and silencing.

A. tumefaciens cells harboring empty pRI 101-AN vector was used along with plants without any treatment as control. All the plants were kept at 24°C, 16 h light and 8 h dark for acclimatization (4-6 days) before infection and maintained at the same conditions throughout the experiment. Samples for metabolic and real-time analyses were collected at 4 and 8 days after infection (DAI).

2.9. Statistical analysis

GC-MS analyses of volatiles and gene expression analysis were performed with five biological and three technical replicates each. Data sets were represented as mean ± standard deviation. Significant differences between control and treated plants for metabolites and gene expression were determined using two-way ANOVA followed by Bonferroni's multiple comparisons test.

3. Results and discussion

3.1. OkBCS shares similarities and ancestry with other sesquiterpene synthases

A transcript (Contig44) from *Ocimum* transcriptome exhibited high sequence similarities with known sesqui-terpene synthase genes. This ORF was cloned from young leaf cDNA of *Ocimum kilimandscharicum* and designated as *OkBCS* (GenBank ID: KP226502). It was 1,629 bp long and encoded a protein of 542 amino acids with 63.63 kDa mass and pI of 5.66. OkBCS shared high similarity with several previously characterized sesqui-terpene synthases, including BCS from *Lavandula angustifolia* Mill. L. (70%) (AGL98419; [16]) and (-)-germacrene D synthase from *Ocimum basilicum* (58%) (Q5SBP6; [17]). Protein sequence alignment of OkBCS

with other BCS's from *Lippia dulcis* Trevir. (J7LJN5; [18]), *Matricaria recutita* L. (I6RAQ6; [19]), *Artemisia annua* L. (Q8SA63; [20]) and *Vitis vinifera* L. (E5GAF4; [21]) revealed presence of several highly conserved regions of sesqui-terpene synthases (Fig. 1A). These included the conserved aspartate-rich region (DDxxD), which is crucial for the substrate binding [22] and'xDx$_6$E' motif for metal cofactor binding [23]. No chloroplast-targeting sequence was identified in OkBCS, which indicated its function as a cytosolic sesqui-terpene synthase. A 'RR(X)$_8$W' motif was present in the N-terminal region of OkBCS and downstream of the N-terminal transit peptide, that is assumed to participate in the ionization of the substrate [24] and is characteristic of majority of the members of subfamilies TPS-a and TPS-b [25]. Another conserved region, 'RxR' motif was also found in OkBCS, which was located 35 amino acids upstream of the DDxxD motif and known to form complex of diphosphate group after substrate ionization [26]. NJ tree placed OkBCS closely to LaBCS, along with cluster of other BCSs and sesqui-terpenes from *Ocimum* (Fig. 1B). Thus, sequence analysis and phylogenetic tree suggested that *OkBCS* could have evolved from a common ancestor more closely related to TPS-a subfamily.

3.2. Heterologous expression and functional characterization of OkBCS

Recombinant OkBCS was purified to homogeneity from the cell-free homogenates by Ni^{2+}- affinity chromatography (Fig. 2A). For OkBCS enzyme assay, purified protein was incubated with farnesyl diphosphate (FPP) and geranyl pyrophosphate (GPP) as substrates [27]. The cyclization mechanism of BCS has been described previously [28]. GC and GC-MS analysis of the assay extract with FPP indicated the presence of one major (94%) and minor (6%) metabolites (Fig.2B). The compounds were identified as β-caryophyllene and α-humulene by

comparing the EI mass fragmentation pattern, retention time and GC co-injection studies with authentic standards (Fig. 2B). Assays with empty vector as control or without FPP as a substrate did not yield any product. OkBCS was not active with GPP as substrate, however, incubation with GPP showed the traces of geraniol (the hydrolyzed product of GPP). OkBCS showed a fairly broad pH range, with maximum near pH 8 (Fig. S1) and required Mg^{2+} as co-factor.

The K_m value of OkBCS with FPP was 125.5 µM and was higher compared to other sesqui-terpene synthases, *viz*., from *Zingiber zerumbet* (L.) Sm. (α-humulene synthase, K_m=32 µM) and *Laurus nobilis* L. (LnTPS3, K_m=43.4 µM) which had about 3-4 times lower K_m values [29,30]. Additionally, similar K_m values were observed from other sesqui-terpene synthases [31–33]. The V_{max} 2.55 $\mu M \cdot s^{-1}$ and k_{cat} 4.06 s^{-1} were also high compared to many other reported sesqui-terpene synthases and were in the typical range of enzymes of the secondary metabolism [32]. The relatively high K_m value suggested a low binding affinity of OkBCS to FPP. Overall, OkBCS resembled in principle reaction parameters and general properties of BCS from *Artemisia annua* [20] and *Lavandula langustifolia* [16]; and belonged to typical class of sesqui-terpene synthases from angiosperms [34].

3.3. Volatile accumulation in different organs among Ocimum species

To survey natural variation of β-caryophyllene, we analyzed volatile profiles of six different tissues from ten different individuals of five *Ocimum* species. Usually, β-caryophyllene is found in nature as a mixture with α-humulene [35]. In all the species of *Ocimum*, both β-caryophyllene and α-humulene were present in a ratio of about 15:1. In *Ocimum tenuiflorum*, *Ocimum kilimandscharicum* and *Ocimum americanum*, β-caryophyllene was the major sesqui-terpene ranging from 6-18% relative abundance (Fig. 3A). Highest levels were found in *Ocimum tenuiflorum* II inflorescence (18%) and in floral and leaf tissues (16%) of *Ocimum tenuiflorum*.

All the tissues of *Ocimum kilimandscharicum* and *Ocimum americanum* had around 6% β-caryophyllene except in root, while 2% in *Ocimum gratissimum* I and II. Trace level (~1%) of β-caryophyllene was found in all the tissues of *Ocimum basilicum* I to IV, and in stem and root of *Ocimum basilicum* III. Abundance of α-humulene was relatively low to the levels of β-caryophyllene in all the analyzed *Ocimum* species across the tissues (Fig. S2). Results of two way ANOVA for β-caryophyllene levels showed a statistically significant variation of 58.44% at $P<0.0001$ in species and 14.8% at $P=0.001$ between tissues types.

3.4. Species and tissue specific mRNA accumulation patterns of OkBCS

Transcript abundance of *OkBCS* in different tissues across five *Ocimum* species indicated a positive correlation between gene expression and metabolite levels in all the species, except *Ocimum tenuiflorum* (Fig. 3B). *OkBCS* showed higher expression in young leaves and inflorescence, while lower expression in mature leaves, flowers and stem. Further across five *Ocimum* species, *OkBCS* showed the highest expression in *Ocimum kilimandscharicum* followed by *Ocimum americanum*, *Ocimum tenuiflorum* and *Ocimum gratissimum* while *Ocimum basilicum* had trace levels. Results of two-way ANOVA showed significant interaction between different tissues and *Ocimum* species (total variance of 5.17%, $P<0.0001$). Despite lower expression of *OkBCS*, β-caryophyllene was higher in almost all the tissues from *Ocimum tenuiflorum*. This could be due to contribution from other TPS in β-caryophyllene production as byproduct [29,36] which lead to overall higher β-caryophyllene level compared to other *Ocimum* species.

3.5. Role of OkBCS in β-caryophyllene biosynthesis

Transient expression using *Agrobacterium* is frequently used as it allows rapid analysis of gene function. Several reports are available where transient expression mediated by *Agrobacterium* was used for studying gene expression [37], gene silencing [38] and protein-protein interaction [39]. To demonstrate *OkBCS* expression patterns and their effect on metabolite levels, transient over-expression and silencing assays were establish in *Ocimum kilimandscharicum* by syringe assisted agro-infiltration method. Changes in metabolites and transcripts were calculated by comparing with values from non-infected control plants at respective stages (Fig. 4A and 4B). Plants infected with empty vector constructs were also used to nullify any effect of tissue injury as wounding might have led to increase in β-caryophyllene levels [40]. In qRT-PCR analysis, silencing effect was more prominent in systemic leaves with 95% reduction in *OkBCS* transcripts against 51% decrease in local leaves at 4 DAI. However, this silencing effect was less at 8 DAI as systemic tissues showed only 45% reduction in transcripts of *OkBCS* while 31% decrease in local tissues. This was consistent with the available reports indicating that the effect withered away upon time during transient expression, owing to the instability of non-integrated T-DNA copies [41]. Plants infected with empty vector constructs had slight increase in transcript levels in local tissues at both stages that could be attributed to wounding (Fig. 4A). Overall, two-way ANOVA followed by Bonferroni's multiple comparisons test suggested significant difference in *OkBCS* transcripts between control and *OkBCS* silenced plants ($P< 0.01$) (Fig. 4A). Further, this silencing effect in transcript abundance was also evident from changes in metabolite levels (Fig. 4B and Table S2). β-caryophyllene level decreased by 20% in silenced plants compared to controls at 4 DAI (Fig. 4B and 4C) with α-humulene

following the similar trend. However apart from these two metabolites, no other terpenes displayed any significant change in their levels at both stages (Table S2).

In *OkBCS* over-expression studies, transcript level was 400% higher than controls on 4 DAI while this was decreased to 250% at 8 DAI in local leaves. The same trend was observed in case of systemic tissues with 160% increase in transcript level at 4 DAI, which reduced to 27% at 8 DAI (Fig 4A). However, metabolite accumulation was not consistent with increase in the *OkBCS* transcript at both the stages. At 4 DAI, β-caryophyllene increased to 22% and 32% in local and systemic tissues, respectively, compared to control plants while it rose to 52% and 56% in local and systemic leaves, respectively at 8 DAI.

4. Conclusion

β-caryophyllene synthase was functionally characterized from *Ocimum kilimandscharicum* for the first time. OkBCS carried out the cyclization of FPP into β-caryophyllene as a major product along with α-humelene as a minor metabolite. *OkBCS* expression variation correlated with metabolite levels among five *Ocimum* species. Agro-infiltration based transient expression assay was established in *Ocimum* that clearly demonstrated the role of OkBCS in β-Caryophyllene accumulation. These findings may pave the way to improve disease resistance in crop plants and enriched medicinal properties.

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Figure Legends

Fig. 1: (A) Amino acid sequence alignment of BCS from *Ocimum kilimandscharicum* (OkBCS, A0E3KJK7) with reported sequences from *Lippia dulcis* (J7LJN5), *Matricaria chamomilla* (I6RAQ6), *Artemisia annua* (Q8SA63) and *Vitis vinifera* (E5GAF4). Different conserved domains are indicated in red. **(B)** Neighbour-joining tree showing phylogeny of OkBCS with other TPSs using MEGA6.

Fig.2: (A) His-tag purified (purified 1) and desalted (purified 2) recombinant OkBCS on 12% SDS-PAGE gel. **(B)** GC separation of authentic standards of (i) β-caryophyllene, (ii) α-humulene (iii) FPP without rOkBCS and (iv) *in vitro* assay products of FPP with rOkBCS; 1. β-caryophyllene and 2. α-humulene.

Fig. 3: (A) β-caryophyllene levels [relative abundance (%)] and **(B)** *OkBCS* expression analyses in different tissues (as shown in different colors) of five *Ocimum* species. Absolute quantification was done using EF-1α as the endogenous control. *Og- Ocimum gratissimum, Ot- Ocimum tenuiflorum, Ok- Ocimum kilimandscharicum, Ob- Ocimum basilicum, Oa- Ocimum americanum.*

Fig. 4: (A) *OkBCS* expression levels determined by qRT-PCR. The values are expressed in comparison to the transcript levels in control. **(B)** β-caryophyllene levels upon *OkBCS* over-expression and RNAi silencing (L-local; S-systemic). Values represent relative change (%) in comparison to β-caryophyllene levels in control. Two way ANOVA followed by Bonferroni's multiple comparisons suggested significant difference between the data at $p<0.001$ (indicated as '***'), $p<0.01$ (indicated as '**'). **(C)** GC chromatograms; empty vector, *OkBCS* over-expressed and *OkBCS* silenced plants in local leaves at 8 DAI. Int. Std. - Linalyl acetate.

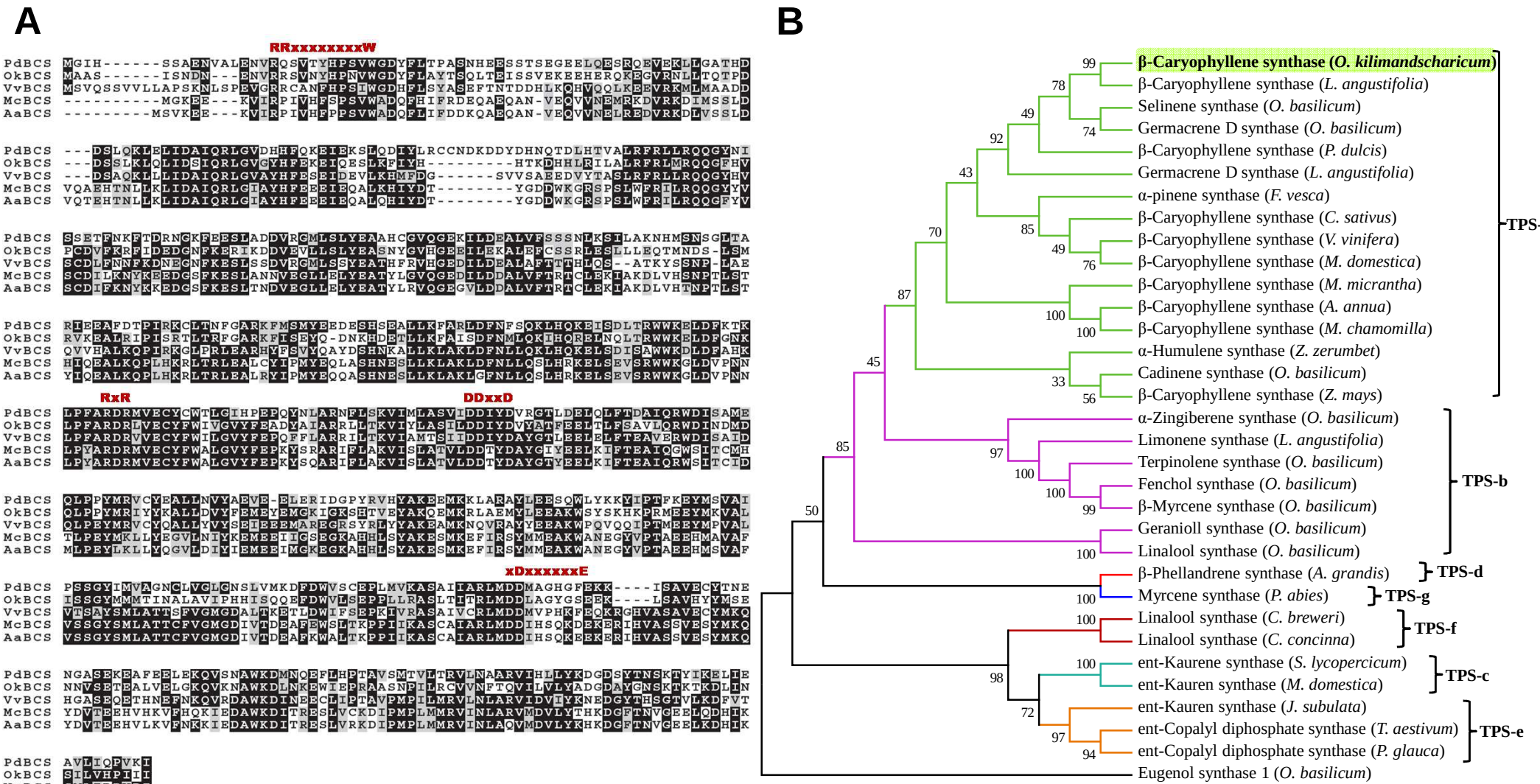

Figure 1

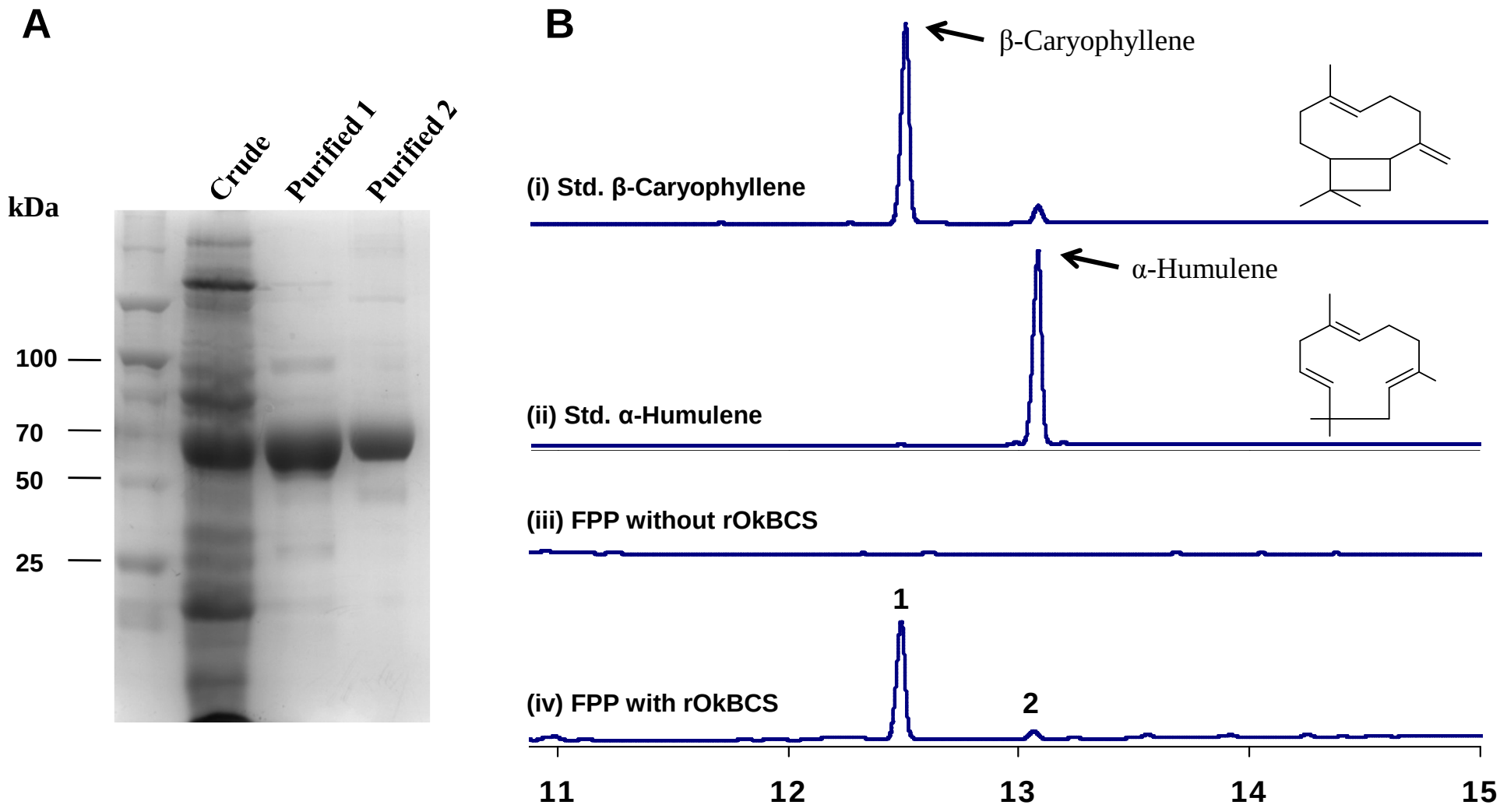

Figure 2

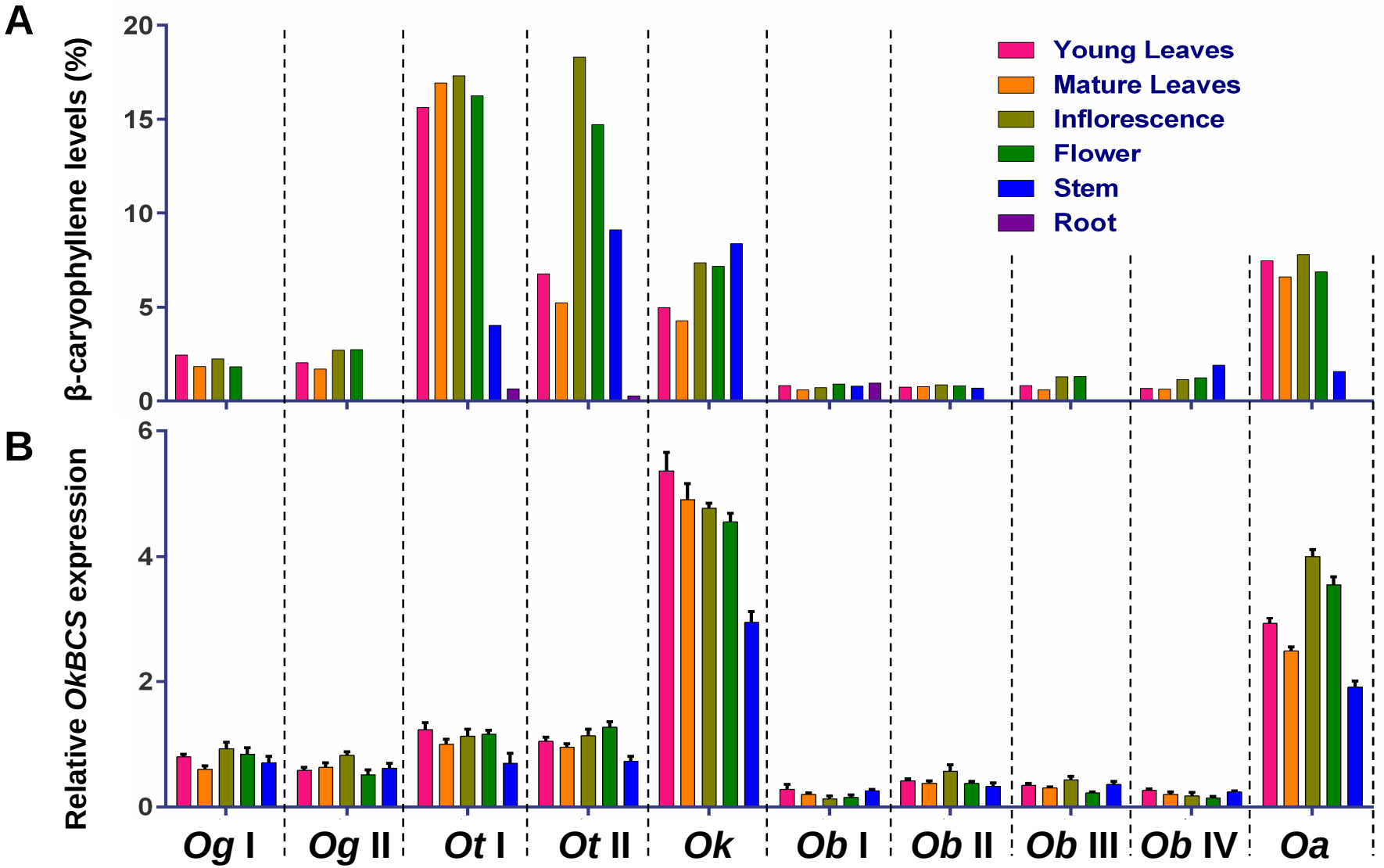
A
β-caryophyllene levels (%)
20
15
10
5
0
Young Leaves
Mature Leaves
Inflorescence
Flower
Stem
Root
B
Relative OkBCS expression
6
4
2
0
Og I
Og II
Ot I
Ot II
Ok
Ob I
Ob II
Ob III
Ob IV
Oa

Figure 3

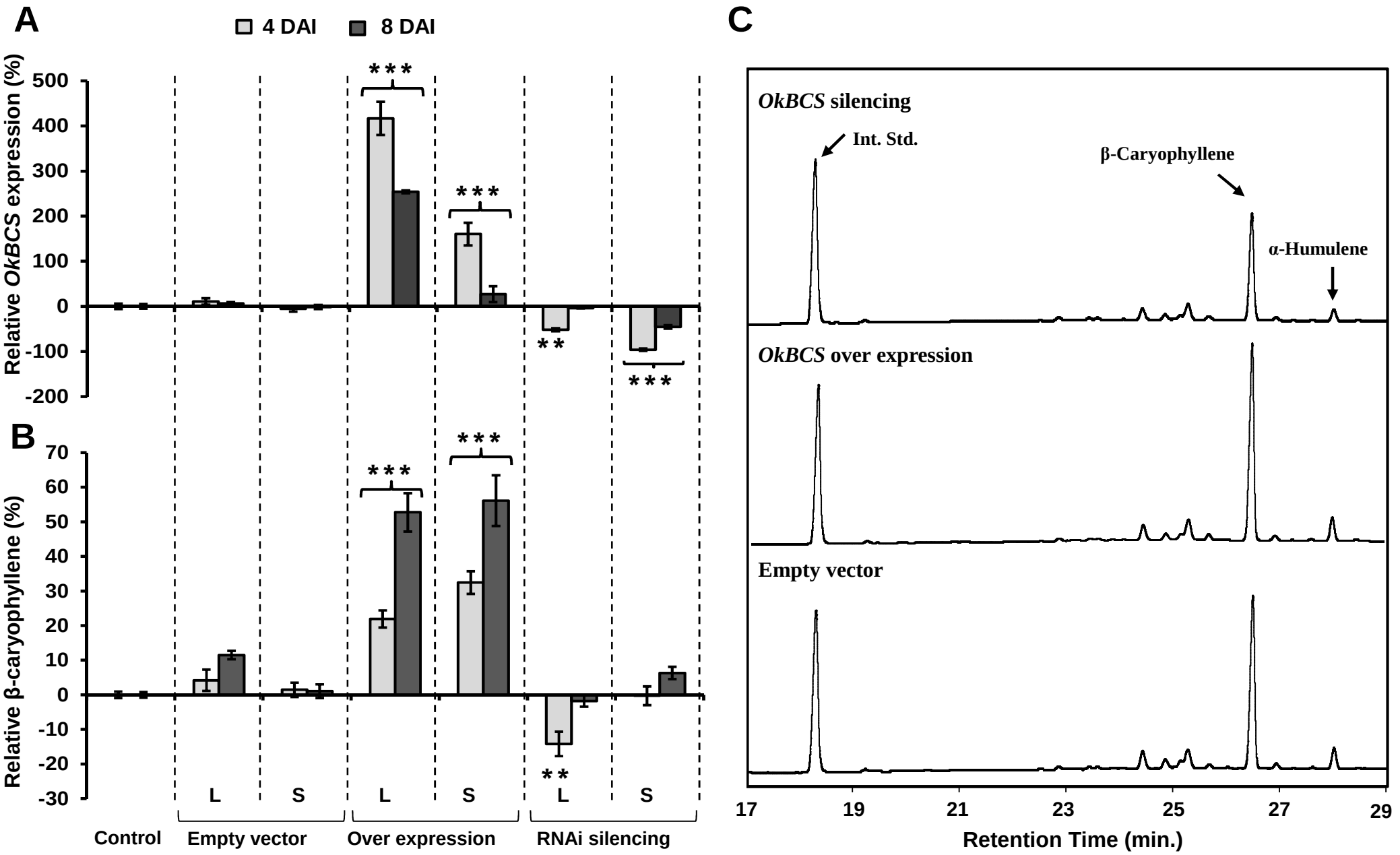

Figure 4

Highlights:

- A new β-caryophyllene synthase is isolated from *O. kilimandscharicum* (OkBCS).
- OkBCS encodes 542 amino acids with mass of 63.6kDa and pI of 5.66.
- Enzyme coverts FPP into β-caryophyllene (94%) and α-humulene (6%).
- *OkBCS* expression correlates with β-caryophyllene levels in tissues of 5 *Ocimum* spp.
- Transient expression of *OkBCS* corroborates its role in β-caryophyllene accumulation.