Isolation and analysis of a multifunctional triterpene synthase promoter region from mangrove plant *Kandelia candel*

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Abstract. Molecular cloning of *Kandelia candel* KcMS gene has previously been cloned and encoded a multifunctional triterpene synthase. In this study, the KcMS gene promoter was cloned through Genome walking, sequenced, and analyzed. A 1,358 bp genomic DNA fragment of KcMS promoter was obtained. PLACE and PlantCARE analysis of the KcMS promoter revealed that there was some regulatory elements in response to environmental signals and involved in the regulation of gene expression. Results showed that four kinds of elements are regulated by hormone binding, namely 2 MeJA-responsiveness elements (CGTCA-motif and TGACG-motif), the ABRE (TACGTG) involved in abscisic acid responsiveness, gibberellin-related GARE-motif (AAACAGA), and the TGA-element (AACGAC) as an auxin-responsive element. Several elements in the KcMS have been shown in other plants to be responsive to abiotic stress. These motifs were MBS (CAACTG), TC-rich repeats, and eight light responsive elements. The KcMS promoter was also involved in the activation of defense genes in plants such as HSE (AAAAATTC) and four circadian control elements (CAANNNNNATC). The presence of multipotential regulatory motifs suggested that KcMS may be involved in regulation of plant tolerance to several types of stresses.

Keywords: Abiotic stress, *cis*-acting elements, gene expression, leaf, terpenoid.

1. Introduction
Mangrove plants are distributed in intertidal tropical and subtropical regions and have long been well known a source of triterpenoids and phytosterols [1-2]. Molecular cloning of *Kandelia candel* KcMS gene has previously been cloned and accumulated a mixture of lupeol, β-amyrin, and α-amyrin. The KcMS, therefore, encoded a multifunctional triterpene synthase, a member of oxidoqualene cyclases (OSCs) gene. The open reading frame (ORF) of KcMS consists of 2286 bp that encodes a 761 amino
acid polypeptide [3]. Molecular mechanism of salinity tolerance in mangrove plants *K. candel* has been reported: mRNA level of *KcMS* was enhanced with salt concentration in both roots and leaves of *K. candel* [4]. This elevated expression of salt-dependent change in mRNA level of *KcMS* was reversible after removal the salinity and transfer to freshwater [5].

Particular emphasis was on the triterpenoid because these compounds were used as biomarkers of organic matter from mangrove as well as lipid input into the estuarine sediments [2]. Furthermore, OSCs have been attracted our consideration because of their possibility to modify the chemical structures of triterpenoids, as well as, their significance as the primarily committed enzymes in the triterpenoid biosynthesis [3].

However, promoters region from mangrove plants and their expressions have not been studied yet. Apart from these metabolic shifts to overcome environmental stresses. Thus, the present study aimed to describe the isolation and analysis of a multifunctional triterpene synthase *KcMS* promoter region from mangrove plant *K. candel*.

2. Materials and Methods

2.1. Plant samples and DNA extraction

Fresh young leaves of *K. candel* were collected at Okukubi River, Okinawa, Japan and used for DNA extraction. Total genomic DNA was extracted from *K. candel* leaves using modified cetyl trimethyl ammonium bromide (CTAB) procedure [6]. The quality of total DNA (1.2 µg) was evaluated using 1% agarose gels and then quantified by UV-Spectrophotometer (Shimadzu, Kyoto, Japan). The material extraction was stored at -20 ºC.

2.2. Isolation of *KcMS* promoter from mangrove plant *K. candel* by PCR-based genome walker

The Universal Genome Walker kit (Clontech, USA) was used to obtain promoter region of a multifunctional triterpene synthase *KcMS* gene as described in Figure 1. For isolation of *KcMS* promoter, the genomic DNA library was digested with *Dra*I (library 1), *EcoRV* (library 2), *Pvu*III (library 3), and *Stu*I (library 4) in separates tubes to make a blunt end. Genome Walker adaptors were ligated to the digests, and the ligated products were used as a template to amplify promoter regions of *KcMS* gene.

![Figure 1. Schematic isolation of *KcMS* promoter used Genome walking technique.](image)

Based on the full-length sequence of *KcMS* gene, for isolation of *KcMS* promoter, two specific oligonucleotide primers: *Kc*-A1 (5’-GATTTCCCTCACACTTGAACCTTCC-3’) and *Kc*-A2 (5’-CTAAACTCCATGTTCGCTTCCCAGG-3’), were synthesized. These primers were used along with two outer adapter primers provided with the kit, AP1 (5’-GTAATACGACTCACTATAGGG-3’) and AP2 (5’-CCCATCCCTCAGGTCGCAATCG-3’). The first PCR was performed using AP1 primer and *Kc*-A1 primer with following PCR conditions: 7 cycles at 94 ºC for 25 s and 72 ºC for 3 min; 35 cycles at 94 ºC for 25 s, 67 ºC for 3 min and a final extension 67 ºC for 7 min. The nested PCR with AP2 and *Kc*-A2 primer was carried out with the first PCR product as template. PCR amplification scheme was 5 cycles for 25 s at 94 ºC and 3 min at 72 ºC, followed by 25 cycles of 25 s at 94 ºC and 3 min at 67 ºC, with a final extension of 7 min at 67 ºC.

2.3. Promoter analysis
The PCR product of promoter fragment was separated using 1% agarose gel GTG and purified by the Suprec-01 filter (Takara Bio Inc). The purified fragment was ligated into a plasmid vector of TOPO TA cloning vector (Invitrogen) and propagated in Escherichia coli, and sequenced by ABI PRISM™ 3100-Avant Genetic Analyzer (Applied Biosystems) with BigdyeR Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequence identification for putative cis-regulatory elements in KcMS promoter was analyzed with PLACE (http://www.dna.affrc.go.jp/PLACE/signalup.html) [7] and PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [8]. The transcription start site in KcMS was predicted online using Neural Network Promoter Prediction (http://fruitfly.org/seq_tools/promoter.html)

3. Results and Discussion

The results will be discussed in two subsections; they are KcMS promoter sequence analysis and the putative functions of important cis-regulatory elements

3.1. KcMS promoter sequence analysis

The promoter region of KcMS gene was cloned from K. candel genomic DNA. A 1,358 bp fragment upstream the start codon of KcMS was obtained (Figure 2). The sequence of the promoter of KcMS gene along with several identified core fragments is shown in Figure 2, which is homologous to the cis-regulatory elements of other plants and of importance for the promoter functions. Two predicted transcription start sites were found in KcMS promoter, at position +493 to +543 with a score of 0.93 and at position +602 to +652 with a score of 0.94 (Figure 2). The promoter sequence has been shown that transcription factors and RNA polymerase bind and regulate the genes [9]. The specific DNA was recognized by transcription factors as cis-acting elements and regulates the gene expression in environmental stimuli [10]. Environmental stresses, including salt stress, therefore, induce the expression of many genes including transcription factors [11] and KcMS gene [4-5].

3.2. The putative functions of important cis-regulatory elements

To elucidate the transcriptional regulation of KcMS promoter, the upstream was isolated and analyzed for various putative cis-acting elements using PLACE and PlantCARE databases. Table 1 shows cis-acting elements of K. candel KcMS promoter. PLACE and PlantCARE analysis of the KcMS promoter revealed that there was some regulatory elements in response to environmental signals and involved in the regulation of gene expression. As displayed in Table 1, four kinds of elements are regulated by hormone binding, namely 2 MeJA-responsiveness elements (CGTCA-motif at position -1341 and TGACG-motif at position +1341), the ABRE (TACGTG, position +753) involved in abscisic acid responsiveness. Furthermore, two gibberellin-related GARE-motifs (AAACAGA, positions -525 and +1061), and two TGA-elements (AACCAG, positions -337 and +1250) as auxin-responsive element MBS also related to ABA-signaling (Figure 2).

The TGACG-motif had been previously reported as the binding site for transcription factors involved in the MeJA signal transduction pathway in barley [12], as two complementary sequences (CGTCA and TGACG) in Catharanthus roseus [13], also found in this study (Figure 2). ABRE cis-acting element (TACGTG) has been reported to be involved in the osmotic, cold, salt, drought, and ABA responsiveness [9-11], suggested that the importance understood regulatory gene in the stress response of cis-acting elements. Besides, GARE-motif was identified as gibberellin (GA) response element (GARE) play an important role in promoting flowering as a factor GA-regulated signaling pathways [15]. Several elements in the KcMS have been shown in other plants to be responsive to abiotic stress. These motifs were MBS (CAACTG), TC-rich repeats, and eight light responsive elements. The KcMS promoter was also involved in the activation of defense genes in plants such as a HSE (AAAAAATTC), involved in heat stress responsiveness and four circadian control elements (CAANNNNATC). The circadian element is known to be late elongated hypocotyls (LHYs) and circadian clock-associated 1 (CCA1) to increase morning expression of genes [14].
Figure 2. The genomic sequence of the KcMS promoter showing various *cis*-acting elements for gene regulation predicted in the PLACE and PlantCARE databases are dashed and marked out.
Table 1. Cis-acting elements of K. candel KcMS promoter from Place and PlantCare databases

| Cis-element     | Sequence (5’-3’) | Function                                           |
|-----------------|------------------|----------------------------------------------------|
| ABRE            | TACGTG           | Abscisic acid responsiveness element               |
| CAAT-box        | CAAAT            | Promoter and enhancer regions                      |
| CAT-box         | GCCATC           | Meristem expression                                |
| CATT-motif      | GCATT            | Light responsive                                   |
| CGTCA-motif     | CGTCA            | MeJA-responsiveness element                        |
| G-box           | CACGTG           | Light response                                     |
| GARE-motif      | AAACAGA          | Gibberellin-responsive element                     |
| GC-motif        | GCCCCGG          | Anoxic specific enhancer-like element              |
| GT1-motif       | GTTTAAT          | Light responsive element                           |
| HSE             | AAAAAATTC        | Heat stress responsive element                     |
| I-box           | GATATGG          | Light responsive element                           |
| MBS             | CAACTG           | Light responsive element                           |
| MNF1            | GTGCC (A/T) (A/T)| Light responsive element                           |
| Skn-1-motif     | GTCAT            | Endosperm regulatory element                       |
| SpI             | GGGCGG           | Light responsive element                           |
| TATA-box        | TATA             | core promoter element around -30 of a transcription start |
| TC-rich repeats | GTTTTCTTAC       | Defense and stress-responsive                      |
| TCT-motif       | TCTTAC           | Light responsive element                           |
| TGA-element     | AACGAC           | Auxin-responsive element                           |
| TGACG-motif     | TGACG            | Salicylic acid (SA)- and MeJA-responsiveness element |
| chs-CMA2a       | GCAATTCC         | Light responsive element                           |
| Circadian       | CAANNNNATC       | Circadian control element                          |
| WBOXATNPRI      | TTGAC            | SA-responsive element                              |

Furthermore, two TGA-elements are presents in KcMS promoter gene. The function of these putative auxin responses remains to be elaborated. Searching the regulatory elements in KcMS promoter such as TATA and CAAT boxes are indications of the functionality of sequence. Thirty-four putative TATA boxes are the type of core-sequence element essential for transcription initiation [9]. Twenty CAAT sequences were predicted upstream from the start site of transcription. Therefore, the presence of numerous putative cis-regulatory motifs is in this OSC promoter region as well as the trans-acting factors still need further investigation.

4. Conclusions
The presence of multipotential regulatory motifs suggested that KcMS promoter region may be involved in regulation of plant tolerance to several types of stresses. The new KcMS promoter region will further deepen research on the regulation and biosynthesis of triterpenoids in mangrove plants.

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