Characterization of Cycloartenol Synthase KcCAS Promoter Region from Mangrove Plant
(Kandelia obovata Sheue, H. Y. Liu & J. Yong)

Mohammad Basyuni1, a), Ridha Wati1, Shigeyuki Baba2, and Hirosuke Oku3

1Department of Forestry, Faculty of Forestry, Universitas Sumatera Utara, Medan, North Sumatera, Indonesia.
2International Society for Mangrove Ecosystems, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan.
3Molecular Biotechnology Group, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan.

a)Corresponding author: m.basyuni@usu.ac.id

Abstract. Cloning of Kandelia obovata (S. L.) Yong (2003) KcCAS gene from young roots (formerly known as Kandelia candei) has previously been reported and encoded a cycloartenol synthase. Fresh leaves of K. obovata that naturally grown in Okukubi River, Okinawa, Japan and used for DNA extraction. In this study, the KcCAS gene promoter was cloned through Genome walking, then sequenced and analyzed. An approximately 1306 bp genomic DNA fragment of KcCAS promoter was obtained using the Universal Genome Walk. PLACE and PlantCARE analysis of the KcCAS promoter revealed that there was a number of regulatory elements in response to environmental signals and involved in the regulation of gene expression. Results showed that five kinds of cis-acting elements are regulated by hormone binding. These are the CREABRE (TAcGTO) involved in abscisic acid responsiveness, gibberelin-related GARE-motif (TCTGTTG), the P-box (CCTTTT) involved in the gibberellin-responsive element, TCA-element (GAGAAGAATA), involved in salicylic acid responsiveness, and TGA-element (AAGGAC) as an auxin-responsive element. Several important cis-acting elements in the KcCAS have been shown in other plants to be responsive to abiotic stress. These motifs were MBS (CAACTG), TC-rich repeats, and seven light responsive elements. The KcCAS promoter was also involved in the activation of defense genes in plants such as Box-W1 (TTGACC), DOCT (CACCACGT), ARE (TGTGTT), and two circadian control elements (CAANNNNATC). The presence of multipotential regulatory motifs suggested that KcCAS may be involved in regulation of plant tolerance to several types of stresses.

Keywords: Kandelia obovata, cis-acting elements, cycloartenol synthase, promoter

INTRODUCTION

Mangrove forest is distributed in the intertidal zone24 of tropical and subtropical climates, and a source of triterpenoids and phytosterols [1, 2]. Molecular cloning of Kandelia obovata KcCAS gene (previously known as Kandelia candei) has previously been reported and encoded a cycloartenol synthase [3]. The KcCAS, therefore, is responsible for phytosterol biosynthesis, a member of oxidoreductase cyclases (OSCs) gene. The open reading frame (ORF) of KcCAS consists of 2277 bp that encodes 758 amino acids [3]. Molecular mechanism of salinity tolerance in mangrove plants K. obovata has been reported: mRNA level of KcCAS was not modulated by salt concentration in the roots and decreased in the leaves of K. obovata [4]. The expression of KcCAS in the roots was not affected by salt stress and was lowered after removal of salinity and transfer of freshwater [5]. By contrast, the expression of KcCAS was reduced by salinity, and this decrease was reversible by subsequent fresh water [5].

Particular reference was on the phytosterols because these compounds were significant to serve as membrane permeability, serve as precursors for steroid hormone, and biomarkers for marine and terrestrial organic matter [6,
It has been reported that the highest mitochondrial target peptide in the possibility of the potential transit peptide was in KeCAS [1]. Furthermore, OSCs have been targeted because of their potential to be transformed into a diverse range of phytosterol product, as well as their significance as the primarily committed enzymes in the sterol biosynthesis [3].

The biosynthesis of phytosterol contributes to the common pathway up to the branching point of 2,3-oxidosqualene. A variety of triterpenoids and phytosterols widely distributed in plants are biosynthesized from a common precursor 2,3-oxidosqualene by the enzyme OSCs. 2,3-oxidosqualene, therefore, locates at the branching point of isoprenoid pathway toward phytosterols or triterpenes biosynthesis [3]. Nonetheless, promoters region of phytosterols from mangrove plants and their expressions have rarely been reported. Aside from these metabolic changes to prevailing over environmental stresses, thus, the present study aimed to characterize the cycloartenol synthase KeCAS promoter region from mangrove plant K. obovata.

MATERIALS AND METHODS

Fresh young leaves of K. obovata were collected at Okutaki River, Okinawa, Japan and used for DNA extraction. Total genomic DNA was extracted from K. obovata leaves using modified cetly trimethyl ammonium bromide (CTAB) procedure [8]. 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 -80 °C.

The Universal Genome Walker Kit (Clontech, USA) was used to obtain promoter region of a cycloartenol synthase KeCAS gene as shown in Fig. 1. For isolation of KeCAS promoter, the genomic DNA library was digested with DdeI (library 1), EcoRV (library 2), PvuII (library 3), and SflI (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 KeCAS gene.

Based on the full-length sequence of KeCAS gene, for isolation of KeCAS promoter, two specific oligonucleotide primers: KeCAS-A1 (5'-GGGAACTGAAGGCCATGAGGAGATC-3') and KeCAS-A2 (5'-GCAGGTAGTTGTTATGTCCTAGGC-3'), were synthesized. Two outer adaptor primers provided with the kit, AP1 (5'-GTAATACGACTCACTATAGGG-3') and AP2 (5'-CCCACATCTCTTCAGAG-GTGCAATGGC-3') were also used. The first PCR was performed using AP1 primer and KeCAS-A1 primer with following PCR condition; seven cycles at 94 °C for 25 s and 72 °C for 3 min, 35 cycles at 94 °C for 25 s, 67 °C for 3 min and a final extension 67 °C for 7 min. The nested PCR with AP2 and KeCAS-A2 primer was carried out with the first PCR product as template. PCR amplification was five cycles for 25 s at 94 °C and 3 min at 72 °C followed by 25 cycles of 25 s at 94 °C and 3 min at 67 °C, with a final extension of 7 min at 67 °C.

The amplification of PCR product of promoter fragment was separated using 2 % 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) and BigDye Terminator version 1.3 Cycle Sequencing Kit (Applied Biosystems). The sequence identification for the presence of cis-regulatory elements in KeCAS promoter was computational analyzed with PLACE [9] and PlantCARE database [10]. The transcription start site in KeCAS was predicted online using Neural Network Promoter Prediction (http://fruitfly.org/seq_tools/promoter.html) [11].

RESULTS AND DISCUSSION

Approximately 1 306 bp genomic DNA fragment of KeCAS promoter was obtained from K. obovata using genome walking method. The promoter region was located at the adjacent start codon ATG as shown in Fig. 1. To further understanding of the transcriptional regulation of KeCAS promoter, many cis-acting elements involved in gene expression and environmental signals were analyzed in PLACE [9] and PlantCARE [10] databases. Table 1 shows five kinds of cis-acting elements are regulated by hormone binding, the ABRE motif (TACGTG, position +235) involved in abscisic acid (ABA) responsiveness, gibberellin-related GARE-motif at position +543 (TCTGTTC), the P-box (CCCTTTTGT, position +1083) involved in gibberellin-responsive element, TCA-element (GAGAAGATA, position +1051) involved in salicylic acid responsiveness, and TGA-element at position +732
(AACGAC) as auxin-responsive element. In another hand, several important elements in the KeCAS have been shown in other plants [9-12] to be responsive to abiotic stress.

The sequence of the promoter of KeCAS gene is depicted in Fig. 2, which is homologous to the cis-regulatory elements and the importance of the promoter functions. The promoter sequence of KeCAS has been shown that transcription factors and RNA polymerase bind to and regulate the genes of basal promoter and basal transcriptional [12]. The specific DNA was recognized by transcription factors as cis-acting elements that regulate the gene expression in environmental stimuli [13]. Environmental stresses, including salt stress, therefore, induce the expression of many genes including transcription factors [14] and KeCAS gene from K. obovata [4, 5].

![Genomic DNA Diagram]

**FIGURE 1.** Schematic isolation of KeCAS promoter region carried out by Genome walking method

<table>
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<tr>
<th>Table 1: Cis-acting elements of K. obovata KeCAS promoter from Place and PlantCare databases.</th>
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<tr>
<td><strong>cis-element</strong></td>
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<td>SUTR Py-rich stretch</td>
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These responsive and regulatory elements to abiotic stress motifs as shown in Fig. 2 were MBS at position +609 (CAACTG), TC-rich repeats at position +1095, and ten light responsive elements (five G-boxes, GAG-motif, GT1-motif, MNF1-motif, l-box, and Sp1 motif). The KeCAS promoter was also involved in the activation of defense genes in plants such as Box-W1 at position +170 (TTGACC), dOCT (CaCGGATC), ARE at position +955 (TGGTTT), and two circadian control elements at position +518 and -686 (CAANNNNTT). The occurrence of
two circadian elements was also reported in isoprene synthase of poplar leaves to increase early expression of promoter elements [15]. Two types of cis-acting element belong to the ABA-dependent pathway, identified in this study: ABRE and MBS are an MYB recognition site [13,14]. The present results indicated the importance understanding of the regulatory genes possibly involved in several stresses such as osmotic, cold, drought, salt, light, and ABA signaling.

![Promoter sequence](image)

**Figure 2.** The sequence of the *KcCAS* promoter depicting various cis-acting elements for gene regulation; predicted is dashed and marked out in a different color. Grey: cis-acting elements conferring high transcription levels. Yellow: cis-acting regulatory element, red: cis-acting element involved in the abscisic acid (ABA) signal; green: cis-acting regulatory element essential for the anaerobic induction, blue: fungal elicitor responsive element, purple: a common cis-acting element in promoter and enhancer regions and cis-acting regulatory element related to meristem expression, and black: gibberellin-responsive element, etc.

As displayed in Table 2, the transcription initiation site in *KcCAS* was predicted using Neural Network Promoter Prediction [10] consisted of three sites in forward strand at position +1 to +51 (C), at position +73 to +123 (A), and position +83 to +133 (A). On the other hand, there are five transcription sites, at position -1063 to -1013 (T),
position -931 to -881 (O), position -583 to 533 (G), position -303 to -253 (G), and -247 to -197 (C). The diversity of transcription start sites was noted within the strand, suggested the importance of this promoter region. Some plant genes have a conserved promoter sequence called the TATA box located 25 to 35 base pairs upstream of the transcription start site, and CAAT box dominated the regulatory elements [16, 17], including in this study. Sixteen CAAT sequences and twenty-four TATA boxes were found in KcCAS promoter from the start site of transcription. Transcription factors bind to the TATA box and initiate the formation of the RNA polymerase transcription complex, which promotes transcription initiation sites [17]. Therefore, the presence of abundant putative cis-regulatory motifs in KcCAS promoter region as well as the trans-acting factors are still needed further research.

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<th>TABLE 2. Promoter prediction of KcCAS (5′) score cutoff 0.80 (transcription start shown in larger font)</th>
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<td>Predictions for the reverse strand (−) of KcCAS</td>
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CONCLUSION

This study confirmed the presence of multi potential regulatory motifs suggested that KcCAS, a cycloartenol synthase may be involved in regulation of plant tolerance to several types of stimuli. The new KcCAS promoter from *K. obovata*, a member of OSCs gene will extend our knowledge on regulation and sterol biosynthesis in the mangrove forest.

ACKNOWLEDGMENT

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