Cloning and Phylogenetic Analysis of Actin Genes from Mangrove Family Rhizophoraceae

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Abstract. Plant actin is one of the most conserved eukaryotic proteins to maintain the basic cellular process. Homology based PCRs method with degenerate primers designed from the conserved amino acid sequences of diverse actin genes have resulted in cloning of three actin genes. Three partial actin genes were cloned from mangrove family Rhizophoraceae, namely leaves of Bruguiera gymnorrhiza, roots of Kandelia candel and leaves of Rhizophora stylosa. The clones termed BgAct1 (944 bp, coding for 273 amino acids), KcAct1 (1083 bp, coding for 273 amino acids), and RsAct1 (894 bp, coding for 273 amino acids), respectively. The deduced amino acid sequences of BgAct, KcAct1 and RsAct1 showed significant similarity (93-96%) to known plant actin genes. These results showed that three fragment genes of BgAct, KcAct1 and RsAct1 encoded actin gene. To observe the relationship of BgAct, KcAct1 and RsAct1 to other plant actin genes, the rooted phylogenetic analysis was constructed. Phylogenetic tree shows that BgAct, KcAct1 and RsAct1 join with Eucommia Act and Pyrus Act cluster, suggesting that our three-actin genes are new members of plant actin genes.

1. Introduction
Higher plants including mangrove plants contain families of actin encoding genes, which are divergent and differently expressed. Plant actin, especially β-actin, are expressed constitutively and involved in basic housekeeping function required for cell maintenance [1]. They are therefore used as endogenous internal standards for normalizing gene expression studies. Actin gene therefore play an important role as internal standard with stable expression under particular conditions, biotic and abiotic stress [2].

Mangroves forests comprise a heterogeneous group of independently derived lineages that are defined ecologically by their location in intertidal zones of tropical and subtropical regions [3]. Mangrove are salt tolerant and it is therefore important to get more insight into the molecular mechanism of salinity tolerance at cellular level.
The actin genes of dicot and monocot plants are more strongly linked than duplications, represents that actin gene is purely from gene family of ancient times [1]. Therefore, cloning and identification more new reference genes will be highly valuable for plant structure and function. Furthermore, the importance of actin gene and its role in plant species including from mangrove plants is poorly understood. However, to our knowledge, study on actin gene in mangrove plants is scarce. Here we report three cDNAs sequence encoding actin gene from mangrove family, Rhizophoraceae, namely *Bruguiera gymnorrhiza*, *Kandelia candel* and *Rhizophora stylosa*. In order to gain some understanding of the pattern of actin gene evolution, phylogenetic analysis also is discussed.

2. Materials and method

2.1. PCR and sequence analysis for actin gene

PCR was performed with a PTC-200 Peltier Thermal Cycler (MJ Research). The PCR reaction products were separated by SeaKem GTG agarose (BMA), purified by SuprecTM-01 (Takara Bio Inc.), ligated to TOPO 10 (Invitrogen), and introduced into electrocompetent *E. coli* (Invitrogen) by Gene Pulser XcelTM (Bio-Rad). Plasmid DNA was extracted by GenEluteTM Plasmid Miniprep Kit (Sigma-Aldrich). Sequencing was carried out by ABI PRISMTM 3100-Avant Genetic Analyzer (Applied Biosystems) using BigdyeR Terminator ver. 1.1/3.1 Cycle Sequencing Kit (Applied Biosystems).

2.2. Total RNA isolation and cDNA synthesis

Total RNA from leaves *B. gymnorrhiza* and *R. stylosa*, and roots of *K. candel* were extracted using CTAB method as described in our previous report [4] with minor modifications to improve the yield and quality of total RNA. Total RNA was dissolved into 20 µl of DEPC treated water (approximately 2-5µg/µl in concentration). The first-strand cDNA was synthesized from 2-5µg of total RNA with Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) with 10 mM dNTP in a total volume of 20 µl by incubating for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC according to the manufacturer’s instruction. After first-strand cDNA synthesis, cDNA was treated with 2 units of RNase H to remove the RNA and used as template.

2.3. Cloning of partial actin gene

Total RNA from leaves of *B. gymnorrhiza* and *R. stylosa* and roots of *K. candel* were reverse-transcribed with 2.5 µM oligo (dT)20 primer to produce a cDNA in total volume of 20 µl by incubating for 5 min at 65 ºC, 1 h at 50 ºC, and 5 min at 85 ºC, and using cloned AMV First-Strand cDNA synthesis Kit (Invitrogen) according to manufacturer’s protocol. The resulting cDNA mixture was diluted with 50 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and directly used as template for the following PCRs. Two degenerate oligonucleotide primers (actinF and actinR) corresponding to the highly conserved amino acid sequence of diverse actin genes [5] were used to amplify the core fragment of actin gene (ActinF = 5’-TACCCNATYGARCACGG-3’ and ActinR = 5’-TTRATRTRCDCKRACRATTTCC-3’ (10 ng)) using Ex TaqTM HS DNA polymerase (Takara Bio Inc.) and 0.2 mM dNTP in a final volume of 50 µl. PCR amplification was carried out for 2 min at 94 ºC, followed by 35 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC and 30 sec at 72 ºC, with a final extension of 8 min at 72 ºC. Three PCR products as core sequences (401, 405, 390 bp) were separated using 1 % agarose SeaKemR GTGR agarose (BMA) and purified by Suprec TM-01 filter (Takara Bio Inc.).

The purified fragments were ligated to a plasmid vector of TOPO 10 (Invitrogen) and propagated in *E. coli*, by Gene Pulser XcelTM (Bio-Rad) and sequenced by ABI PRISMTM 3100-Avant Genetic Analyzer (Applied Biosystems) with BigdyeR Terminator ver. 1.1/3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequence revealed the presence of two types of core fragments (KcAct1 and RsAct1). The number of clones sequenced for *BgAct1*, *KcAct1* and *RsAct1* genes was 4, 2 and 4 respectively.

Based on the sequences of above three types of core fragment, specific primers to each gene were designed to amplify the 3’-ends of cDNAs by RACE method [6] using GeneRacerTM Kit (Invitrogen).
The primers used for 3′-RACE were as follows: BgACT-S1 (5′-ATGTACGTGCCCAGTCCAGG-3′), BgACT-S2 (5′-GTAAGATACCTACGAC-3′); KcACT-S1 (5′-GCCATGTACGTGCCATCCAC-3′), KcACT-S2 (5′-CCCCATCTGCTGAGCGTGAA-3′); and RsAct-S1 (5′-GCCAGCTATGTACGTTGCCAG-3′).

The PCR products of the 3′-ends of each gene were cloned into a TOPO 10 plasmid vector, and propagated in *E. coli* for sequencing. In the case of 3′-RACE, four clones of each gene were sequenced in both strands. The partial DNA sequence of BgAct1, KcAct1 and RsAct1 were obtained.

2.4. Similarity and phylogenetic analysis of amino acid sequence of plant actin genes

The amino acid sequences were aligned and similarity scores were obtained using the FASTA ver. 3.4t26 [7] of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan). The best score of results are shown in Table 1. Phylogenetic analysis of deduced amino acid alignment from plant actin genes was conducted with CLUSTAL W ver. 1.83 [8] of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan) followed by drawing with TreeView, ver. 1.6.6 [9] based on a neighbor-joining method. Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree [10].

The DDBJ/GenBank/EMBL accession numbers of the sequence of used this analysis are as follows: X16280 (*Oryza sativa* Ra1), U76191 (*Pisum sativum* PEAc9), AF386514 (*Pyrus communis*), AB032361 (*Mimosa pudica* actin isoform B), AF288226 (*Setaria italica*), AY742219 (*Saccharum officinarum* Saactin), AB491932 (*Bruguiera gymnorrhiza* BgAct1), AB491931 (*Kandelia candel* KcAct1), AB573024 (*Rhizophora stylosa* RsAct1), BT003847 (*Arabidopsis thaliana* Act-12), AY360221 (*Malva pusilla*), EF418792 (*Populus trichocarpa*), AY825362 (*Chorispora bungeana* CbACTIN2), AY653160 (*Eucommia ulmoides* ACT1), AF172094 (*Picea rubens*), EF418791 (*Ricinus communis*), AB491932 (*Bruguiera gymnorrhiza* BgAct1), AB491931 (*Kandelia candel* KcAct1), AB573024 (*Rhizophora stylosa* RsAct1), BT003847 (*Arabidopsis thaliana* Act-12), AY360221 (*Malva pusilla*), EF418792 (*Populus trichocarpa*), AY825362 (*Chorispora bungeana* CbACTIN2), AY653160 (*Eucommia ulmoides* ACT1), AF172094 (*Picea rubens*), DQ252512 (*Solanum tuberosum*), AY305732 (*Gossypium hirsutum*).

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3. Results and Discussions

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![Alignment](image)

**Figure 1.** Sequence alignment of the deduced amino acid from *B. gymnorrhiza* (BgAct1), *K. candel* (KcAct1), *R. stylosa* (RsAct1). Identical amino acid residues of three proteins are shaded and dashes indicate alignment gaps. The DDBJ/GenBank/EMBL accession numbers: AB491932 (BgAct1), AB491931 (KcAct1), and AB573024 (RsAct1).
3.1. Cloning of actin gene fragment from Rhizophoraceae family

The actin gene of *B. gymnorrhiza* (BgAct1), *K. candel* (KcAct1) and *R. stylosa* (RsAct1) for internal standard were amplified based on the highly preserved regions of known plant actin genes, as described previously [5]. The core sequence of BgAct1, KcAct1 and RsAct1 (401, 405 and 390 bp) in length, respectively were cloned into a TOPO 10 vector (Invitrogen). Four clones for KcAct1 and two clones for BgAct1 were extended to 3'-end by 3'-RACE method as described previously [6].

The partial sequence of BgAct1, KcAct1 and RsAct1 were 944, 1083 and 894 bp, respectively. These DNA sequences encoded 277 amino acids residues for three cases. KcAct1 and BgAct1 shared 97.6% identities in their amino acid sequence, and 88.6% in their DNA sequence. RsAct1 showed high similarities in amino acid (98.3%) and 90.9% in DNA sequence with BgAct1.

The deduced amino acid sequence of BgAct1, KcAct1 and RsAct1 showed significant similarity to known plant actin genes (Table 1). The deduced amino acid sequences of BgAct, KcAct1 and RsAct1 showed significant similarity (93-96%) to known plant actin genes. These results showed that three fragment genes of BgAct, KcAct1 and RsAct1 encoded actin gene. KcAct1 showed high similarity (93.9-96.0%) to the actin gene from a variety of plant species. The similarities of BgAct1 with plant actin genes were also high (95-96.6%). These results showed that three BgAct, KcAct1 and RsAct1 encoded actin gene, and can be reasonably used as internal standard for real-time PCR.

| Clones | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   | 14   | 15   | 16   | 17   | 18   |
|--------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 1      | 100  | 95   | 94   | 95   | 95   | 94   | 95   | 95   | 94   | 96   | 94   | 95   | 95   | 96   | 95   | 94   | 94   | 94   |
| 2      | 95   | 100  | 94   | 95   | 95   | 95   | 95   | 96   | 95   | 94   | 95   | 95   | 96   | 95   | 94   | 96   | 95   | 94   |
| 3      | 94   | 94   | 100  | 95   | 95   | 96   | 93   | 95   | 95   | 96   | 96   | 95   | 95   | 96   | 95   | 95   | 96   | 95   |
| 4      | 94   | 95   | 95   | 95   | 98   | 98   | 93   | 97   | 94   | 98   | 94   | 98   | 96   | 97   | 95   | 95   | 93   | 95   |
| 5      | 95   | 95   | 95   | 98   | 100  | 98   | 94   | 97   | 94   | 99   | 94   | 98   | 97   | 97   | 95   | 93   | 96   | 96   |
| 6      | 95   | 95   | 96   | 98   | 98   | 100  | 94   | 97   | 96   | 98   | 95   | 98   | 97   | 97   | 96   | 95   | 94   | 95   |
| 7      | 94   | 95   | 93   | 93   | 94   | 94   | 100  | 94   | 93   | 94   | 94   | 94   | 94   | 94   | 93   | 94   | 93   | 94   |
| 8      | 95   | 96   | 95   | 97   | 97   | 97   | 94   | 100  | 94   | 97   | 95   | 96   | 95   | 96   | 94   | 95   | 95   | 96   |
| 9      | 95   | 95   | 94   | 94   | 94   | 96   | 93   | 94   | 100  | 95   | 95   | 95   | 95   | 96   | 95   | 94   | 94   | 96   |
| 10     | 94   | 95   | 96   | 98   | 99   | 98   | 94   | 97   | 95   | 100  | 95   | 99   | 96   | 97   | 95   | 95   | 93   | 96   |
| 11     | 96   | 94   | 96   | 94   | 94   | 95   | 94   | 95   | 95   | 95   | 100  | 95   | 95   | 96   | 95   | 95   | 95   | 95   |
| 12     | 94   | 95   | 96   | 98   | 98   | 94   | 97   | 94   | 97   | 95   | 100  | 96   | 97   | 95   | 95   | 93   | 96   | 96   |
| 13     | 95   | 95   | 95   | 96   | 97   | 97   | 94   | 96   | 95   | 96   | 95   | 96   | 100  | 97   | 96   | 94   | 93   | 95   |
| 14     | 95   | 95   | 95   | 97   | 97   | 97   | 94   | 96   | 95   | 97   | 95   | 97   | 100  | 96   | 94   | 93   | 94   | 93   |
| 15     | 96   | 96   | 95   | 95   | 96   | 94   | 96   | 95   | 95   | 96   | 95   | 96   | 96   | 100  | 95   | 96   | 95   | 96   |
| 16     | 95   | 95   | 95   | 95   | 95   | 94   | 95   | 94   | 95   | 95   | 94   | 94   | 96   | 100  | 97   | 98   | 97   | 98   |
| 17     | 94   | 94   | 95   | 93   | 93   | 94   | 93   | 94   | 93   | 95   | 93   | 93   | 95   | 97   | 100  | 96   | 96   | 98   |
| 18     | 94   | 94   | 95   | 95   | 96   | 95   | 94   | 96   | 94   | 96   | 95   | 94   | 96   | 98   | 96   | 100  | 96   | 100  |

3.2. Phylogenetic analysis of plant actin

Three mangrove actin sequences in this work was aligned with 15 existing actin sequences (Table 1). The amino acid alignment was used to generate phylogenetic tree. To observe the relationship of BgAct1, KcAct1 and RsAct1 to other plant actin genes, the rooted phylogenetic analysis was constructed. The phylogenetic tree shows that BgAct1, KcAct1 and RsAct1 join with *Eucommia* Act and *Pyrus* Act cluster, suggesting that our three genes encoded actin genes (Figure 3). The present study indicates that BgAct1, KcAct1 and RsAct1 form a new branch in cluster, suggesting that both genes are new members of plant actin genes.

The phylogenetic analysis of BgAct1, KcAct1 and RsAct1 actin genes showed that (Figure 3), despite the ubiquitous distribution of actin multigene family in plant kindom, most of dicot actin genes including
our genes clustered together on the tree. The clustering of the mangrove actin genes suggests that these
sequences may have involved in the evolution of plant dicots and they are functionally conserved [1].

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**Figure 2.** Phylogenetic tree of plant actin genes including *Bruguiera BgAct1*, *Kandelia KcAct1*, and *Rhizophora RsAct1* from mangrove family Rhizophoraceae. The phylogenetic tree was constructed with the neighbour-joining method of the CLUSTAL W program. The indicated scale represents 0.1 amino acid substitutions per site. Numbers indicate bootstrap value from 1000 replicates.
4. Conclusions
The present study indicates that $BgAct1$, $KcAct1$ and $RsAct1$ form a new branch in the cluster, suggesting that the three genes are new members of plant actin genes. The sequence data analysis of mangrove actin genes to improve our understanding about structure of actin genes in mangrove.

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