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Isolation and characterization of *WRKY* genes in coconut (*Cocos nucifera* L.)

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Abstract

WRKY proteins are plant-specific transcriptional factors associated with regulation of defense responses to both biotic and abiotic stresses. In the present study, degenerate PCR primers were designed to the highly conserved *WRKY* DNA binding domain and these were used to isolate putative *WRKY* genes from coconut. Six DNA fragments were isolated using five pairs of degenerate primers and these were cloned. Sequencing of these cloned fragments was done and their amino acid sequences deduced. Homology search of the deduced amino acid sequences against non-redundant GenBank protein database revealed significant sequence similarity of two of the cloned fragments to known *WRKY* genes. The other four cloned fragments showed homology to known stress responsive genes. Cluster analysis based on neighbour-joining method was carried out using the putative coconut *WRKY* genes with other known *WRKY* genes. These results demonstrate that PCR amplification with appropriately designed degenerate primers is an efficient approach for cloning *WRKY* genes.

Key words : Coconut, *WRKY* genes, degenerate primers, PCR, cloning

Introduction

Areas of serious concern for coconut production are the sensitivity of the coconut palm to biotic and abiotic stress conditions. While fungal, bacterial, viral and viroid pathogens contribute substantially to losses in coconut, phytoplasma causing lethal yellowing in Mexico and root (wilt) disease in India represents the most devastating pathogen in coconut. With reference to abiotic stress, sensitivity to drought is a highly important factor, since it limits coconut propagation to the coastal areas where rainfall prevails. Thus, breeding in coconut has to aim for high yielding hybrids with tolerance or resistance to drought and biotic stresses.

Identification of regulatory genes or signaling components in defense and stress responses is one of the most critical steps leading to elucidation of plant defense and stress tolerance mechanism. There are several strategies for isolation of candidate genes. The conserved domains of different genes offer possibility of amplifying DNA fragments analogous to stress responsive genes by PCR using degenerate primers for these conserved motifs.

The *WRKY* proteins are a superfamily of transcriptional regulators, which are involved, in various physiological programs, including biotic and abiotic stress responses as well as development processes (Li *et al.*, 2004; Pnueli *et al.*, 2002; Johnson *et al.*, 2002). The name of the *WRKY* family itself is derived from the most prominent feature of these proteins, the *WRKY* domain, constituted by about 60 amino acid residues. In this *WRKY* domain, a conserved *WRKY*GQK sequence is followed by a C2H2- or C2HC- type of zinc finger motif (Eulgem *et al.*, 2000). All *WRKY* proteins contain either one or two copies of this highly conserved domain. The fully sequenced *Arabidopsis* genome reveals 72–74 members of the *WRKY* gene group in the genome. These can be divided

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into three groups with several subgroups on the basis of their *WRKY* domains and their phylogenetic clades (Eulgem *et al.*, 2000; Dong *et al.*, 2003).

WRKY genes encode transcription regulators with diverse functions that have been important for plant development and defense responses. Expression analyses of the *Arabidopsis thaliana WRKY* gene family indicated that 49 members of the gene group were differentially regulated in plants infected with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* or treated by salicylic acid (Dong *et al.*, 2003). Studies indicate that *WRKY* genes play an important role in the signaling cascade of innate immunity in *Arabidopsis* (Asai *et al.*, 2002; Li *et al.*, 2004) as well as in other plant species such as tobacco (Chen and Chen, 2000; Liu *et al.*, 2004) and rice (Guo *et al.*, 2004). Induction of *WRKY* genes has been observed in the desert legume *Retama raetam* by drought stress (Pnueli *et al.*, 2002) and in *Nicotiana tabacum* by wounding (Hara *et al.*, 2000). Evidence is accumulating that *WRKY* genes may be involved in development and metabolic processes, such as in the seed coat and trichome development of *Arabidopsis* (Johnson *et al.*, 2002), the somatic embryogenesis of orchard grass (Krassimira and Conger, 2002), in the gibberellin signaling pathway in rice aleurone cells (Zhang *et al.*, 2004), carbohydrate anabolism (Sun *et al.*, 2003) and in the regulation of sesquiterpene synthesis (Xu *et al.*, 2004). As such, the phylogenetic analyses of *WRKY* genes can serve as a useful guide for studying their roles in plants.

Borrone *et al.* (2004) reported the use of degenerate primer pairs designed to specifically amplify *WRKY* gene fragments directly from cocoa genomic DNA. With the above background and considering the potential of DNA markers, the present study was undertaken to design degenerate primers for *WRKY* transcription factors, and to clone and sequence *WRKY* transcription factors from coconut using the designed degenerate primers.

Materials and Methods

Primer design

The nucleotide and protein sequences pertaining to *WRKY* transcription factors were retrieved from the NCBI. Sequences were extracted both in GenBank and FASTA format. Sequence conversion and separation was done by using SEQVERTER programme. Multiple sequence alignment of protein sequence was done by offline program CLUSATX. Coding region was selected with BIOEDIT program on the basis of GenBank information and only coding region of nucleotide was used for further analysis. All three open reading frames for the coding region of nucleotide sequences were also analyzed with WINGENE program. After multiple alignment, protein conserved domain position in nucleotide sequence was determined using BIOEDIT program. Nucleotide sequences coding for conserved domain amino acids were selected for oligomer designing. Oligomer sequence was selected with minimum degeneracy and high G-C content. The primers were checked for their quality (GC per cent, annealing temperature, presence of dimers) using the software FASTPCR programme. The degenerate primers designed to amplify putative *WRKY* transcription factors in coconut are given in Table 1.

Table 1. Details of degenerate primers designed and used to amplify putative coconut *WRKY* transcription factors

S.No.	Primer	Sequence (5'-3')
1.	*WRKY* F 1	TACGAGAGGAGCTAAACAGAG
	WRKY R 1	TTTTGTCCGTATTTCCTCC
2.	*WRKY* F 2	AGATGGATTTCAATGGAGG
	WRKY R 2	CATCCCCTTCAGAAGCATTTGG
3.	*WRKY* F 3	TACCAATGGCGCAAGTACGG
	WRKY R 3	GGTTGTGCTCGCCCTCGTA
4.	*WRKY* F 4	ATGGATCCGTGGATTAGCACCCAG
	WRKY R 4	TCAATCCTTGGTCGGCGAGAGCTC
5.	*WRKY* F 1	TACGAGAGGAGCTAAACAGAG
	WRKY R 2	CATCCCCTTCAGAAGCATTTGG

Plant materials

Genomic DNA of drought resistant accession, *Pallikkara Tall I,* was used for the isolation of *WRKY* transcription factors gene sequences. DNA was extracted from spindle leaves following the method of Upadhyay *et al.* (1999) with slight modifications.

Standardization of PCR parameters

Initially, the optimum annealing temperatures were determined for each primer pair using gradient PCR. Once optimized, the PCR reaction was conducted in volumes of

20 μl containing 50 ng genomic DNA, 0.2 ìM each of forward and reverse primers, 50 ìM of each dNTPs (M/s Bangalore Genei Pvt. Ltd., Bangalore), 1X buffer (10 mM Tris-Hcl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) and 0.5 Units of *Taq* DNA polymerase

(M/s Bangalore Genei Pvt. Ltd., Bangalore).

PCR amplifications were performed on a Eppendorf gradient thermal cycler with a PCR profile of 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min at the different annealing temperatures optimized and 1 min at 72°C with a final extension for 15 min at 72°C.

Agarose gel electrophoresis of amplified products

After amplification, the PCR products were resolved in 1.2 per cent agarose gel using 1X TBE buffer, stained with ethidium bromide, visualized in gel documentation system (Alpha Imager™1200, M/s Alpha Innotech Corp., CA, USA) and results documented. The sizes of the amplified fragments were estimated by comparison with a 100 bp ladder (M/s Bangalore Genei Pvt. Ltd., Bangalore).

Isolation of DNA fragments from agarose gel

The PCR fragments were excised from the agarose gel and purified using Perfectprep® Gel Cleanup Kit (M/s Eppendorf). For elution of DNA fragments, 1.2 per cent low melting agarose was used since gels from low melting agarose exhibit low background fluorescence using ethidium bromide staining. The band corresponding to the desired DNA fragment was cut using a sterile scalpel. Care was taken to avoid much exposure to UV on a UV transilluminator. The agarose piece was treated as described in the kit. The purified DNA was then eluted from the membrane and the eluted DNA fragments were stored at -20 °C.

Cloning of the eluted fragments

Cloning of the eluted fragments was carried out using the InsT/A clone™ PCR Product Cloning Kit (M/s MBI Fermentas Inc., USA), which is a convenient system for one-step cloning of PCR-amplified DNA fragments. Ligation of PCR amplified purified DNA fragment was performed using pTZ57R/T vector (Figure 1) as described in supplier's manual. Vector (pTZ57R/T) and inserts were taken in 1:3 ratio. Ligation was conducted in volumes of 30 μl containing 3 μl of plasmid vector pTZ57R/T DNA (0.165μg, 0.18 pmol ends), 4 μl of eluted PCR fragment (approx. 0.54 pmol ends), 3 μl of 10X ligation buffer, 3 μl of PEG 4000 solution and 1 μl of T4 DNA Ligase (5 Units). The ligation mixture was incubated at 22°C overnight.

Fig. 1 Multiple alignment of deduced amino acid sequence of Coc TR3A with other related sequences

Transformation of *E. coli* strains and selection of recombinant clones

Transformation was carried out using TransformAid™ Bacterial Transformation System. After transformation, the cells were finally plated on pre-warmed LB- agar plates [ampicillin (100 ppm) / IPTG (100 ppm) / X-gal (160 ppm)] and incubated overnight at 37 °C. The recombinant clones were identified by blue/white colony selection.

Clone analysis by PCR

Colony PCR was carried out for direct analysis of the positive transformants.

One colony was picked up and resuspended in 20 μl of the PCR mixture. The reaction mixture was incubated for 5 min at 94°C to lyse the cells and inactivate the nucleases. PCR amplification was carried out and the PCR products visualized by agarose gel electrophoresis.

Isolation of recombinant plasmid DNA

Highly pure plasmid DNA was obtained by means of GenElute™ Plasmid Miniprep Kit (M/s Sigma).

PCR amplification of recombinant plasmid DNA

PCR was carried out using the recombinant plasmid DNA as template. The PCR products were analyzed on 1.5 per cent agarose gel.

Restriction digestion of recombinant plasmid DNA

For releasing the insert, the choice of restriction endonucleases for digestion was made after checking the restriction map of the corresponding gene using the software RESTRICTION MAPPER (version 3.0) (http://www.restrictionmapper.org). Double digestions of the plasmid DNA was performed with the restriction enzymes *Eco* RI and *Bam* HI (M/s Fermentas) according

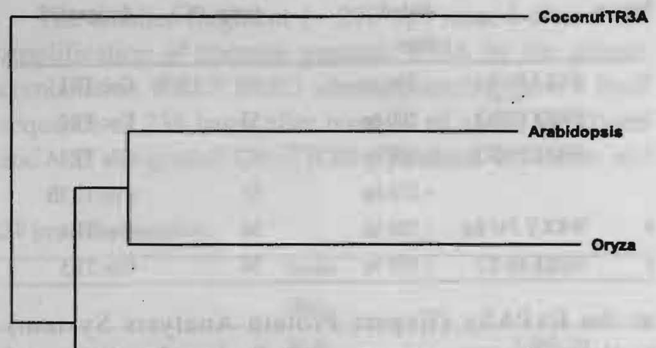


Fig. 2 Phylogenetic tree based on neighbour-joining method of deduced amino acid sequence of Coc TR3A with other related sequences. The scale bar represents a distance of 0.1 amino acid substitutions per site.

to the manufacturer's instructions. Normally, 0.1-10 µg of the plasmid DNA was restricted by 1-8 Units of the restriction endonucleases. Specific enzyme buffer provided with the enzyme were used to achieve best results. Incubation time was 2-16 hours at 37°C. After incubation, the restriction endonucleases were denatured by incubating the mixture at 72 °C for 10 min. The digested DNA was analyzed by agarose gel electrophoresis.

Sequencing of cloned fragments

The recombinant plasmids were sent for sequencing to M/s First Base Asia, Singapore. The sequencing was done from one end of the vector with M13 primer using BigDye® Terminator v3.1 Sequencing Kit and analyzed on ABI PRISM® 377 Genetic Analyzer.

Sequence analysis

The nucleotide sequences from the M13 forward primer was screened for the presence of vector contamination using VecScreen (Altschul *et al.*, 1997). The contaminated region was cut using the software WINGENE (version 2.31). The amino acids were deduced from the nucleotide sequences in all possible frames using the translate option at the ExPASy (Expert Protein Analysis System) proteomics server at the Swiss Institute of Bioinformatics. The resulting amino acids were subjected to similarity search using a local alignment search algorithm, BLAST (Altschul *et al.*, 1997). The scores of alignment were computed with reference to block substitution matrix (BLOSUM). The result of the homology search is detailed as maximum identity for increased length of subject sequences, higher positive values and decreasing E-values. Lower the E-value, the more similar the sequences in terms of homology to the previously reported similar sequences in the database.

Table 2. Band size of fragments eluted from gel after amplification of coconut genomic DNA with different degenerate primers and the name designated.

S. No.	Primer	Band size eluted (bp)	Annealing temp. (°C)	Name designated
1	*WRKY* F1/R1	~ 250 bp	53	Coc TR1
2	*WRKY* F2/R2	~ 700 bp	53	Coc TR2
3	*WRKY* F3/R3	~ 300 bp	57	Coc TR3A
		~ 270 bp	57	Coc TR3B
4	WRKY F4/ R4	~ 700 bp	50	Coc TR4
5	*WRKY* F1/R2	~ 550 bp	50	Coc TR5

Evolutionary relationships among the sequences were charted out by cluster analysis. Amino acid sequences deduced from nucleotides sequences were aligned using T-COFFEE programme (Notredame *et al.*, 2000). A genetic matrix was calculated from the alignment data and analyzed with the neighbour-joining method (Saitou and Nei, 1987). The phylograms were constructed using the PHYLIP phylogenetic tree tool (Felsenstein, 1989).

Results and Discussion

Gene amplification

Amplification of *WRKY* genes in coconut was done using coconut genomic DNA as template and five degenerate primer pairs. Initially, gradient PCR was carried out to select the optimum annealing temperature for PCR. All the five primers gave good amplification. The annealing temperatures optimized for the different primer pairs are given in Table 2. The optimized annealing temperatures were used to amplify putative *WRKY* genes in coconut. The different putative *WRKY* genes amplified in coconut using the five *WRKY* degenerate primer pairs is given in Plate 1.

Elution of the PCR fragments

The PCR amplified fragments were electrophoresed on a 1.2 per cent low melting agarose gel and required band sizes were eluted. Primer *WRKY* F4/R4 and *WRKY* F1/R1 gave multiple bands. For these primers, the expected band size or the strongest bands were eluted (Table 2). One primer combination *WRKY* F3/R3 amplified two strong bands (Plate 1) and both were eluted. After elution, the fragments were run in a 1.2 per cent agarose gel. Plate 2 depicts the eluted putative *WRKY* fragments amplified in coconut. The DNA concentration was around 50-75 ng μl^{-1} invariably for all the fragments.

***Transformation of the ligated product into* E. coli**

The eluted products were ligated with pTZ57R/T. *E. coli* (DH5α) was transformed with the ligated recombinant pTZ57R/T vectors carrying inserts. Colonies started to emerge from 16th hour. Combinations of blue and white colonies were obtained confirming successful transformation.

Colony PCR

The agar plates containing the transformed colonies were screened for recombinant positive clones. White colonies were picked up and were grown in LB broth containing ampicillin. The recombinant colonies were screened for the presence of inserts by colony PCR. For comparison, genomic DNA samples were also used separately for PCR. After PCR, the amplicons were electrophoresed in agarose gel. There was amplification of expected size in all the recombinant clones. Plate 3 depicts amplification of expected sizes for the fragment Coc TR4 amplified by the primer pairs *WRKY* F4/R4.

Table 3. Summary of homology search performed for deduced Coc TR3A peptide sequence.

S. No.	Accession no.	Definition	Score(Bits)	E-value
1	AY341856	*Oryza sativa* WRKY16 mRNA, complete cds	65.7	1.00E-12
2	AY740531	*Capsicum annuum* WRKY-c mRNA, complete cds	74.3	7.00E-12
3	AF439274	*Retama raetam* WRKY-like drought-induced protein mRNA, complete cds	61.6	2.00E-11
4	AY323128	*Glycine max* putative WRKY-type DNA binding protein mRNA	61.6	2.00E-11
5	NM_105598	*Arabidopsis* WRKY57 mRNA, complete cds	70.3	3.00E-11
6	AY547498	*Nicotiana benthamiana* WRKY1 mRNA, partial cds	59.7	1.00E-10

Checking the presence of insert in the recombinant plasmid by PCR

The plasmids isolated from the recombinant clones were checked for the presence of insert by PCR. The recombinant plasmids were amplified by the respective gene specific primers. All the recombinant clones exhibited specific amplification of expected size confirming the presence of the insert.

Checking the presence of insert by restriction digestion

Double digestion with restriction endonucleases was carried out to release the inserted DNA fragments from the recombinant plasmids. On resolving the digested mixture on a 1 per cent agarose gel, the inserts of expected sizes were released (Plate 4). This confirmed that the plasmids contained the inserts.

***In silico* analysis of the nucleotide sequences**

The recombinant plasmids, for which the confirmation tests were performed, were sent for automated sequencing.

Sequence analysis of Coc TR3A

The larger fragment cloned after PCR amplification of coconut genomic DNA by the primer combination *WRKY* F3/R3 after sequencing gave a final sequence of 263 bases after removal of vector backbone and was designated Coc TR3A (GenBank accession no. DQ861408). Homology search of deduced amino acid sequences of Coc TR3A with the reported sequences available in public databases gave 489 hits. Some of the results obtained in the form of hits are given in Table 3. Coc TR3A showed homology to *WRKY* transcription factors from *Oryza sativa, Capsicum annuum, Retama raetam, Glycine max, Arabidopsis thaliana, Nicotiana benthamiana.*

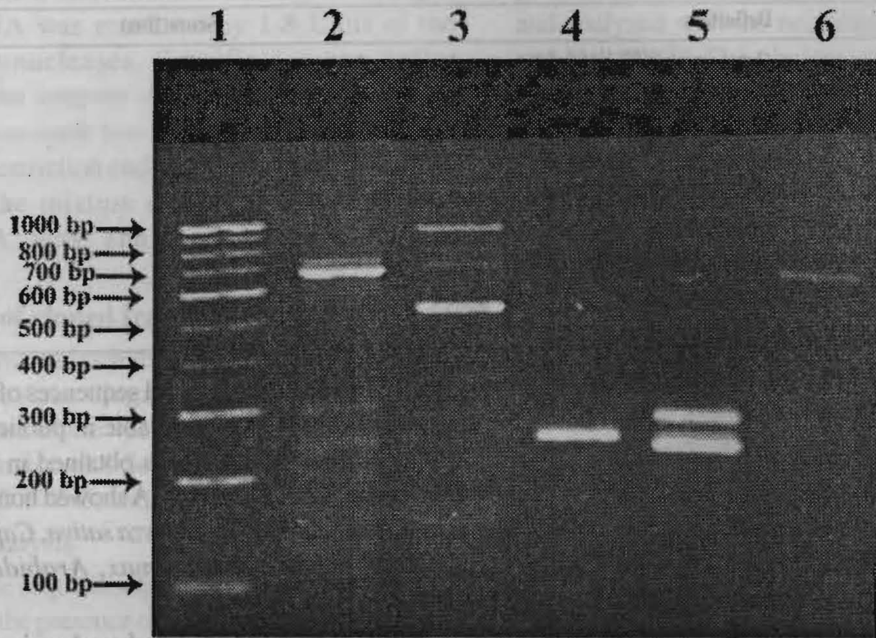
Multiple alignment of deduced amino acid sequence of Coc TR3A with related sequences is given in Figure 1. A phylogenetic tree was constructed based on neighbour-joining method of deduced amino acid sequence of Coc TR3A with other related sequences (Fig. 2). Five major clusters were obtained. Coc TR3A was found to form a distinct cluster.

Sequence analysis of Coc TR3B

The smaller fragment (~ 270 bp) cloned after PCR amplification of coconut genomic DNA by the primer combination *WRKY* F3/R3 after sequencing gave a final sequence of 238 bases after removal of vector backbone and was designated Coc TR3B (GenBank accession no. DQ861409). Homology search of deduced amino acid sequences of Coc TR3A with the reported sequences available in public databases gave 748 hits. Some of the results obtained in the form of hits are given in Table 4. Coc TR3B showed homology to WRKY transcription factors from *Nicotiana tabacum, Arabidopsis thaliana, Zea*

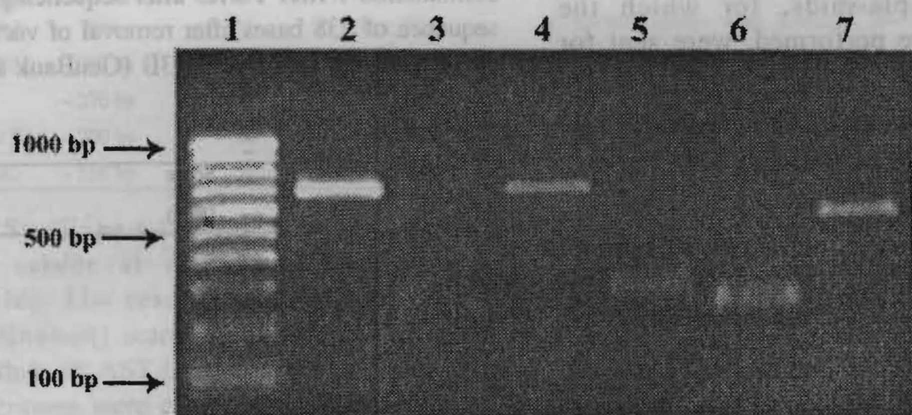
Table 4. Summary of homology search performed for deduced Coc TR38 peptide sequence.

S. No.	Accession no.	Definition	Score (Bits)	E-value
1	AB041520	*Nicotiana tabacum* mRNA for WRKY transcription factor NtEIG-D48, complete cds	90.4	7.00E-26
2	AY062950	*Arabidopsis thaliana* putative WRKY-type DNA binding protein (At2g23320) mRNA, complete cds	87.7	2.00E-25
3	AY940680	*Zea mays* WRKY1 mRNA, complete cds	89	6.00E-25
4	AY514044	*Capsella rubella* WRKY transcription factor 11 (WRKY11) mRNA, complete cds	85.4	1.00E-24
5	AY077758	*Physcomitrella patens* WRKY transcription factor 1 (WRKY1) mRNA, complete cds	84.5	4.00E-24



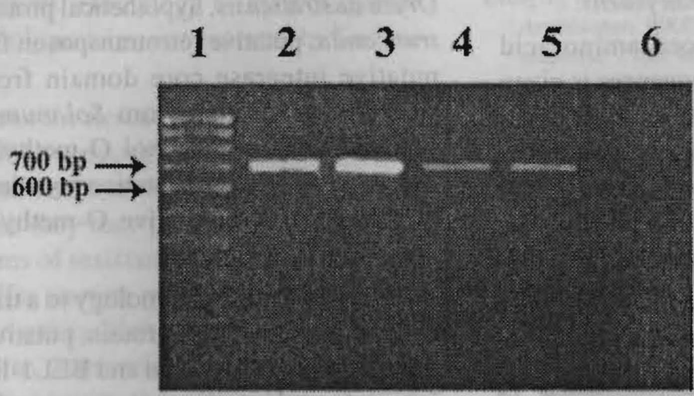
Lane 1: 100 bp ladder **Lane 2: *WRKY* F4/R4**
Lane 3: *WRKY* F1/R2 **Lane 4: *WRKY* F1/R1**
Lane 5: *WRKY* F3/R3 **Lane 6: *WRKY* F2/R2**

Plate 1. PCR amplified putative *WRKY* genes in coconut



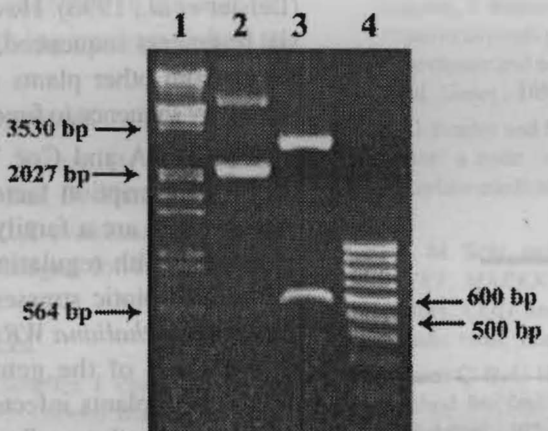
Lane 1: 100 bp ladder **Lane 2: *WRKY* F4/R4**
Lane 3: *WRKY* F1/R1 **Lane 4: *WRKY* F2/R2**
Lane 5: *WRKY* F3/R3 A **Lane 6: *WRKY* F 3/R3 B**
Lane 7: WRKY F1/R2

Plate 2. Eluted putative WRKY fragments after electrophoresis in 1.2 % agarose gel.



Lane 1: 100 bp ladder
Lane2, 3: Colonies
Lane 4,5: Genomic DNA
Lane 6: Negative control

Plate 3. Colony PCR to check the presence of insert, Coc TR4.



Lane 1: Lambda DNA *Eco* RI/*Hin*d III double digest.
Lane 2: Undigested plasmid.
Lane 3: Plasmid digested with *Eco* RI and *Bam* HI.
Lane 4: 100 bp ladder.

Plate 4. Restriction digestion of recombinant plasmid for checking the presence of insert, Coc TR5.

mays, *Capsella rubella* and *Physcomitrella patens*.

Multiple alignment of deduced amino acid sequence of Coc TR3A with related sequences is given in Figure 3. A phylogenetic tree was constructed based on neighbour-joining method of deduced amino acid sequence of Coc TR3B with other related sequences (Fig. 4). Five major clusters were obtained. Coc TR3B was found to cluster with *WRKY* transcriptional factors from *Zea mays*.

Fig. 3 Multiple alignment of deduced amino acid sequence of Coc TR3B with other related sequences

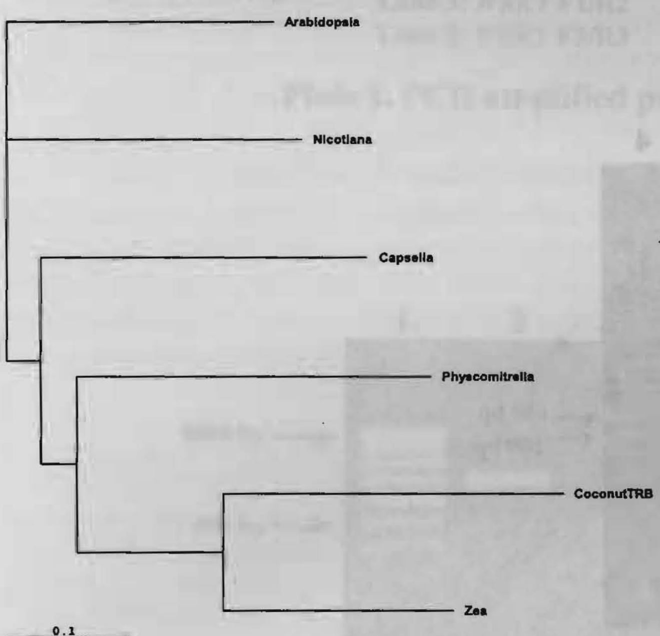


Fig. 4 Phylogenetic tree based on neighbour-joining method of deduced amino acid sequence of Coc TR3B with other related sequences. The scale bar represents a distance of 0.1 amino acid substitutions per site.

Sequence analysis of Coc TR1, TR2, TR4 and TR 5

Coc TR1 showed homology to hypothetical protein, putative retroelement pol protein, splicing co-activator subunit protein Epstein-Barr virus EBNA-1-like protein and BKRF1 encoding EBNA-1 like-protein from *Oryza sativa*. Coc TR2 showed homology to a polyprotein from *Oryza australiensis*, hypothetical protein from *Medicago truncatula*, putative retrotransposon from *Oryza sativa*, putative integrase core domain from *Oryza sativa*, putative polyprotein from *Solanum demissum* (wild potato), putative catechol O-methyltransferase from *Arabidopsis thaliana*, putative myosin-like protein from *Oryza sativa and* putative O-methyltransferase from *Arabidopsis thaliana*.

Coc TR 4 showed homology to a transcription factor, BKRF1 protein, CRK1 protein, putative retrotransposon protein from *Oryza sativa* and BEL1-like homeodomain 5 protein and some other proteins from *Arabidopsis thaliana*. Coc TR5 showed homology to putative cyclic nucleotide-gated ion channel and translation initiation factor from *Arabidopsis thaliana*, major antigen-like protein from *Salsola kali (Russian thistle)*, phosphatidylinositol-4-phosphate 5-kinase 1 precursor from *Oryza sativa and* NADH dehydrogenase subunit 4 from *Corsinia coriandra*.

In the present study, degenerate primers were designed and used to amplify putative *WRKY* transcriptional factors in coconut. Six genomic fragments were isolated and sequenced. A PCR-based approach for cloning heterologous genes may result in amplification products of no functional significance (Leister *et al.*, 1996). However, in this study, out of the six fragments sequenced, two were related to WRKY genes from other plants and the remaining four were related by sequence to functional stress responsive genes.

Coc TR3A and Coc TR3B showed homology to *WRKY* transcription factors from other plant species. *WRKY* genes are a family of regulatory genes that are associated with regulating defense responses to both abiotic and biotic stresses. Expression analyses of the *Arabidopsis thaliana WRKY* gene family indicated that 49 members of the gene group were differentially regulated in plants infected with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* or treated by salicylic acid (Dong *et al.*, 2003). *WRKY* genes are also known to play important roles in the signaling cascade of innate immunity in *Arabidopsis* (Asai *et al.*, 2002; Li *et al.*, 2004) as well as in tobacco (Chen and Chen, 2000; Liu *et al.*, 2004) and rice (Guo *et al.*, 2004). Induction of *WRKY* genes has been observed in the desert legume *Retama raetam* by drought stress (Pnueli *et al.*, 2002).

Deduced amino acid sequence of Coc TR4 and Coc TR2 showed homology to putative retroelements. Putative retroelement pol polyproteins have been shown to co-localize locally with disease resistance genes in *Arabidopsis* and poplar (Lescot *et al.*, 2004) and in apricot (Soriano *et al.*, 2005). The deduced amino acid sequence of Coc TR 1 and Coc TR2 showed homology to putative polyproteins. Homology of apricot disease resistance genes to polyproteins was reported by Soriano *et al.* (2005).

This study is one of the first attempts to isolate, clone and characterize *WRKY* transcription factors in coconut. The isolation and characterization of more of these genes is important because they may provide clues about the complex mechanisms of resistance to biotic and abiotic stress. They also offer starting points for direct cloning of stress responsive genes. Furthermore, cloned genes can be transferred to other related palm species like arecanut and oil palm to study the resistance mechanism in completely different genetic backgrounds.

The pair of degenerate primers designed and utilized in this study could be applied to isolate and characterize WRKY genes from other plant species as the WRKY domain is highly conserved. Also, primers could be designed on the divergent DNA sequence regions to facilitate genetic mapping of the cloned *WRKY* sequences and an assessment of their potential linkages with biotic/abiotic stress resistance genes in coconut once a mapping population segregating for drought tolerance becomes available. These markers could also be converted into sequence tagged site (STS) markers and supplement the limited SSR markers presently available in coconut for association mapping for identifying markers linked to biotic/abiotic stress tolerance.

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