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The Construction of Plant Expression Vector harbouring *Carica Papaya* L. *WRKY* Gene in *Escherichia coli*

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ABSTRACT

Carica papaya is a well-liked and economically important fruit with outstanding nutritional and medicinal values. Its susceptibility to abiotic stress which affects the growth and harvest, causes significant yield loss to farmers. In recent years, significant progress has been made to understand the genes that play critical roles in abiotic stress response, especially some transcription factor (TF) encoding genes. Among all TFs, WRKY TF gene family is one of the best-studied TFs involved in various stress responses. To date, only limited information on functionally characterised WRKY TFs is available for *C. papaya*. The aim of this study was to produce a recombinant construct harbouring *WRKY* gene in pGEM®-T Easy cloning vector. The presence of a DNA band of the expected size of 465 bp on agarose gel electrophoresis indicated that *WRKY* gene was successfully amplified from all treated samples. DNA sequencing analysis revealed that the amplified sequence isolated from the treated samples were closely related to *Carica papaya* species with 97% similarity. Following transformation, 4 out of 5 colonies that were randomly selected showed the *WRKY* gene had been successfully inserted into pGEM®-T Easy vector and transformed into *E. coli*. In future, the *WRKY* gene from pGEMT-*WRKY* recombinant construct will be cloned into

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the plant expression vector pCAMBIA 1304 prior to transformation in the plant. The success of demonstrating the *WRKY* gene towards the response in abiotic stress will enable us to produce stress tolerant transgenic crops under unfavourable conditions via genetic engineering for sustained growth.

Keywords: Abiotic stress, carica papaya L., recombinant, salinity, WRKY transcription factor

INTRODUCTION

Papaya is a well-liked and economically important fruit crop in many countries including Malaysia due to its high nutritive value, production potentiality and many pharmacological benefits (Oliveira & Vitória, 2011). As a common climacteric fruit, papaya suffers many problems including susceptibility to abiotic environmental factors such as drought and high salinity, which could hamper its production worldwide. Up to date, traditional plant breeding has not been proven to be successful in mitigating abiotic stress problems. Hence, understanding how the papaya responds to these stresses at the molecular level could provide more effective strategy to tackle the problems.

Several significant attempts have been conducted to enhance abiotic stress resistance in crops. One of the successful attempts is by regulating the expression of a number of stress-responsive transcription factors (TFs) genes such as TFs from the bZIP, NAC, AP2 and WRKY. Many of these TFs have been shown to play significant roles in abiotic stress particularly to enhance plant adaptation to drought and salt stresses (Ma et al., 2019; Ullah et al., 2018). Among all TFs that have been studied so far, the *WRKY* TF gene family is among the well-known classes of plant TFs (Song et al., 2018; Xie et al., 2018). The earliest *WRKY* gene, known as SPF1 was characterised from sweet potato (Ishiguro & Nakamura, 1994). Since then, a number of WRKY proteins with various functions in many plants have been widely described, specifically playing role in biotic and abiotic stresses (Bai et al., 2018; Liu et al., 2019, 2016), as well as in the regulation of hormones (Chen et al., 2012; Johnson et al., 2002; Lagacé & Matton, 2004; Song et al., 2010).

Lately, the functional characterisation of WRKY proteins in response to abiotic stresses in plants was extensively studied. For example, overexpression of OsWRKY11 and OsWRKY42 in transgenic rice seedlings showed enhanced drought tolerance (Shen et al., 2012; Wu et al., 2009). Similarly, WRKY25 and WRKY26 from *Arabidopsis thaliana* exhibited substantially increased tolerance to heat stress when overexpressed (Li et al., 2011). Other than these two examples, various *WRKY* genes had also been characterised from other plants. For instance, overexpression of SpWRKY1 and GhWRKY41 from tomato and cotton, respectively, had shown remarkable improvement in salt and drought resistance in transgenic tobacco (Chu et al., 2016; Li et al., 2015). More *WRKY* genes

including TaWRKY2 and TaWRKY19 (wheat), VaWRKY14 (grapevine) and PeWRKY83 (moso bamboo) showed increased resistance to drought and/or salt stresses, respectively, when overexpressed in Arabidopsis (Niu et al., 2012; Wu M. et al., 2017).

While the research on the response of WRKY proteins to biotic stress has been widely reported, little information is available to understand the mechanism of WRKY proteins in plant abiotic stress (Niu et al., 2012). However, it is well known that abiotic stress such as high salinity could cause major damage in plants. Under stress conditions, the accumulation of second messengers such as ROS and IP3 modulates cytoplasmic Ca^{2+} and protein signaling pathways, resulting in stress-responsive gene expression and physiological changes in the plant (Huang et al., 2012; Xiong et al., 2002).

In addition, far less information is available to understand the function of WRKY proteins in *C. papaya*. As a major tropical crop consumed worldwide, various abiotic stresses such as drought, salinity and extreme temperature have caused a major hiccup in the papaya industry. Hence, the functional mechanisms on how *C. papaya* responds to abiotic stress need to be identified and genetic engineering could be one of the solutions to achieve this aim. Based on the genome-wide analysis of the *WRKY* gene family in *C. papaya* reported earlier by Pan & Jiang (2014), one of the *WRKY* candidate genes designated as TF807.3 was found to be involved in response to low temperature, drought, wound and PRSV pathogen. However, there has yet been any reports on the functionality of this *WRKY* gene in salinity stress. The current study reports the construction of recombinant construct harbouring *WRKY* gene in pGEM®-T Easy vector and the transformation into *Escherichia coli*. In the future study, the pCAMBIA 1304 plant expression vector harbouring WRKY gene will be constructed prior to transformation into *Arabidopsis thaliana*. The salt stress tolerance will be investigated to determine its response towards salt stress conditions. The success of demonstrating the response of *WRKY* gene towards the abiotic stress will enable us to produce stress tolerant transgenic crops to sustain its growth under unfavorable conditions.

MATERIALS AND METHODS

Plant Materials and Stress Treatment

Seedlings of *C. papaya* L. 'Sekaki' variety purchased from the local supermarket were grown under controlled greenhouse conditions, with a cycle of 16 h/day with three replications. For salt treatment, 30-day-old seedlings with four to five leaves were treated with 200 mM NaCl and leaves were harvested at 24 h following the stress treatment. The control used in this study was the plants treated with only water. Fresh leaves were harvested from each treated and control plants, then rapidly frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. All the experiments were performed independently in triplicate.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from leaves samples using a modified CTAB-based method (Rogers & Bendich, 1994). The quantity and quality of the RNA were examined using NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, USA). The RNA was sequentially treated with DNase I (Invitrogen™, USA) to remove genomic DNA. One microgram of RNA was used for cDNA synthesis using QuantiTect® Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to obtain a whole sequence of WRKY using the first-strand cDNA of *C. papaya* as a template. A pair of gene-specific primers, WRKY4_F: 5′-CCATGGATGGAGAAGTACCAAATTCTTTTCCCAGAC-3′ and WRKY4_R: 5′-AGATCTTTATACAATCAGGTTGAGCGATATTACAAT-3′, were designed based on the *C. papaya* WRKY coding sequence (GI 186140666) to amplify the full-length cDNA sequence. The *Nco*1 and *Bgl*II restriction sites were included at the 5′ end of the WRKY4_F and WRKY4_R respectively, to facilitate construction into plant expression vector pCAMBIA 1304 in the future. The *WRKY* was amplified using GoTaq® Flexi DNA Polymerase (Promega, USA) in a total volume of 25 µL in a thermal cycler (Biorad, USA) with the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of amplification (95°C for 1 min, 56°C for 1 min, and 72°C for 1 min) and a final extension at 72°C for 3 min. The resulting PCR products with an expected size of 465 bp was examined by agarose gel electrophoresis, then purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. The gel-purified DNA was later confirmed by DNA sequencing.

Bioinformatic Analysis of *CpWRKY*

The *CpWRKY* gene sequence was extracted from the local papaya database (GI 186140666) using BLAST with the CDS of *CpWRKY* as a query. BLASTn was performed to search for maximum identity and the number of bases of aligned sequence.

Cloning into pGEM®-T Easy Vector

The purified PCR product and pGEMT®-T Easy vector were digested with *Nco*1 and *Bgl*II restriction endonucleases. The digested PCR product was later cloned into pGEM®-T Easy vector according to the manufacturer's instructions and subsequently transformed into chemically competent *E. coli* DH5α cells using the heat shock method. In order to confirm the successful transformation of recombinant *E. coli* harbouring WRKY, colony PCR was performed using GoTaq® Green Master Mix (Promega, USA) in a total volume of 25 µL. The WRKY4 forward and reverse primers were used to amplify the transformed

gene with the same amplification conditions as described above. The positive recombinant *E. coli* harbouring *CpWRKY* were kept prior to further experiment.

RESULTS AND DISCUSSION

Previously, the functions of plant WRKY transcription factors were extensively reported to play a role in biotic stress responses (Li et al., 2006; Matsushita et al., 2012; Qiu & Yu, 2008; Shen et al., 2007; Xu et al., 2006). In recent years, a number of studies demonstrated that WRKY proteins play important role in abiotic stresses, such as drought and salinity (Chen et al., 2012; Jiang et al., 2012; Zhu et al., 2019). Although WRKY TFs have been reported in many plants, their roles in abiotic stresses are not well known in *Carica papaya*. Therefore, it is of interest to investigate if the WRKY protein from *C. papaya* would function in abiotic stress particularly salinity, as other WRKYs from other crops have been demonstrated to be functional in important crops such as rice, maize and wheat (Cai et al., 2014; Niu et al., 2012; Shen et al., 2012; Wu et al., 2009). To achieve the main aim of this study, WRKY4 primers were designed based on the *WRKY* gene sequence (GI 186140666), obtained from genome-wide analysis in *C. papaya* which has been reported earlier (Pan & Jiang, 2014). This sequence was chosen in this study because it has been shown to be involved in response to low temperature, drought, wound and PRSV pathogen. However, there has yet to be any report on the functionality of this *WRKY* gene in salinity stress. In this paper, we report our preliminary study on the cloning of *C. papaya WRKY* gene in pGEM®-T Easy vector.

Prior to amplification, a *WRKY* gene was isolated from *C. papaya* L. 'Sekaki' that had been treated with 200 mM NaCl and designated as *CpWRKY4*. Following treatment, total RNA was extracted from salt-treated and non-treated (control) plants. Intact total RNA extracted from these *C. papaya* leaves showed 28S rRNA band intensity about twice that of the 18S rRNA (Figure 1). *CpWRKY4* gene was amplified from salt-treated *C. papaya* leaves cDNA by RT-PCR. As a result from agarose gel electrophoresis, the PCR products amplified from the cDNA using WRKY4 primers showed a DNA band of the expected size of 465 bp (Figure 2).

A total of 4 PCR products (Figure 2 Lanes 1 – 4) were sent for sequencing and DNA analysis confirmed that these amplified sequences isolated from the treated samples were closely related to *C. papaya* WRKY sequences reported by Pan & Jiang (2014) with 97% similarity (Figure 3). Based on the results generated in NCBI database, this WRKY TF also showed close identity with other plant species including cotton, squash and grapevine, with 89%, 85% and 84% similarities, respectively (Figure 4). These results indicated that a completed sequence and deduced amino acid sequences of this gene showed homology with WRKY domain-containing proteins from other plants.

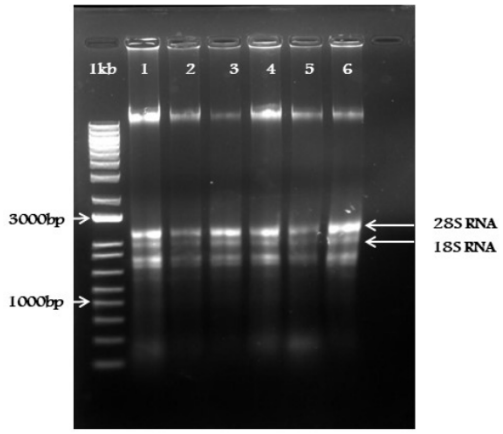

Figure 1. Total RNA extracted from treated and non-treated *C. papaya* following salt treatment. (Lanes 1 – 2: non-treated plant; Lanes 3 – 6: treated plants). 1kb: 1-kb DNA ladder (Fermentas). Molecular sizes are indicated.

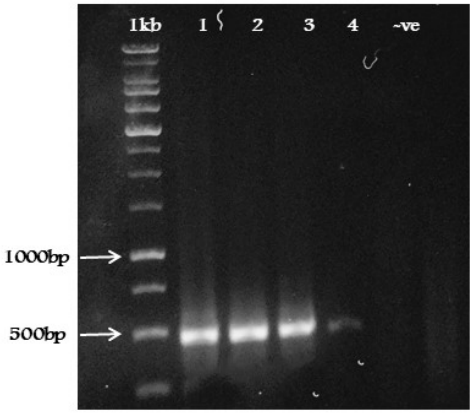

Figure 2. The amplification of full coding sequence of *CpWRKY4* gene using WRKY4 primers (Lanes 1 – 4: treated plants), resulted in the expected amplified product of 465 bp; lane -ve: non-treated plant (control); 1kb: 1 kbp DNA ladder (Fermentas). Molecular sizes are indicated.

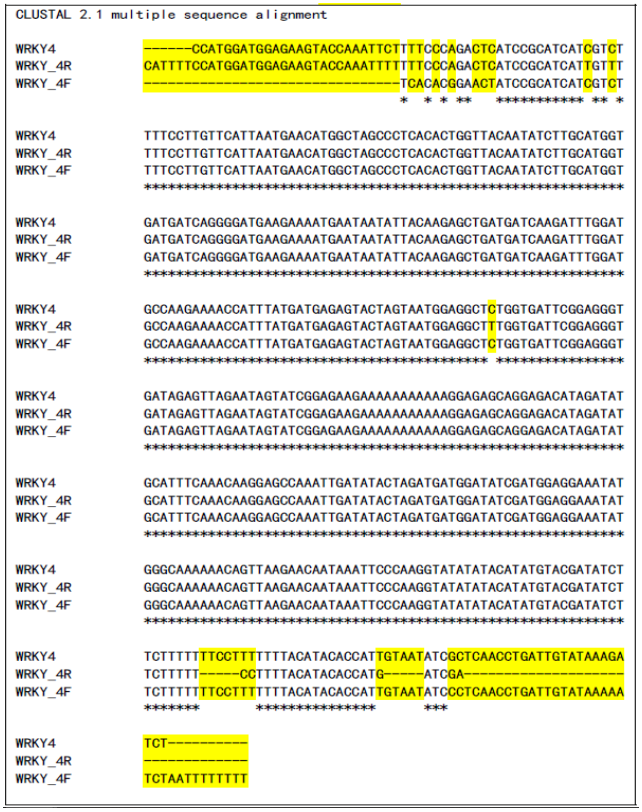

Figure 3. Comparison of the nucleotide sequences of the coding regions of WRKY from *Carica papaya.* The difference in nucleotides are highlighted in yellow and deletions are indicated by dashes.

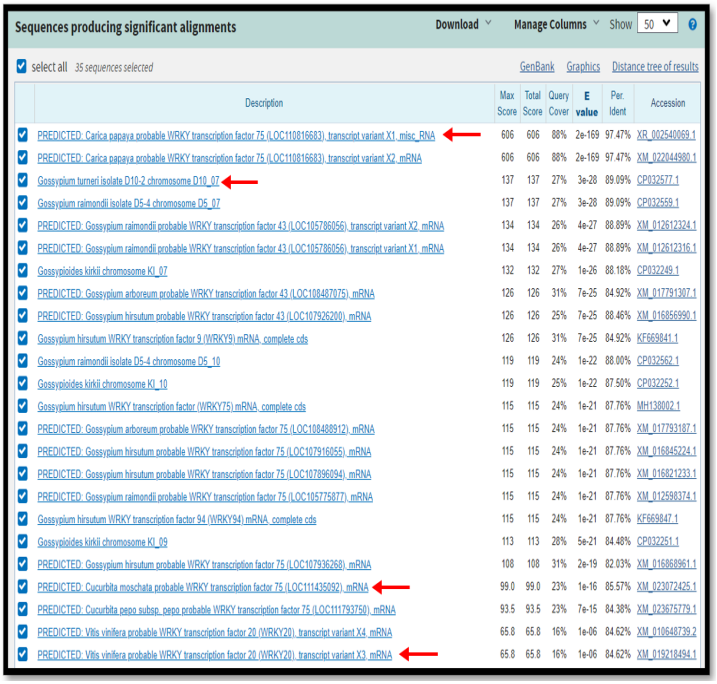

Figure 4. An excerpt of BLAST result, indicating top hits with significant homology to *CpWRKY4* gene from different plant species (indicated in red arrow).

The purified PCR product was cloned into pGEM®-T Easy vector and transformed into *E. coli* DH5α. Following transformation, five colonies were selected and the successful transformation was confirmed by using colony PCR using WRKY4 primers. The results showed that all the colonies except for colony 1 contained the correct WRKY amplified fragment, as indicated by the presence of DNA band of the expected size of 465 bp (Figure 5). In total, 4 out of 5 chosen colonies possessed the desired recombinant *WRKY* gene and these positive clones were selected to be cloned into the plant expression vector, pCAMBIA 1304 in a future experiment.

As a proof of concept, the plant recombinant construct harboring pCAMBIA1304_ *CpWRKY* will be transformed and expressed in a model plant *Arabidopsis thaliana* before generating transgenic *C. papaya* in the future. This is particularly important to investigate whether salt tolerance could be improved and gain further insights into the detailed mechanism of *CpWRKY* in response to salt stress conditions. The success of producing transgenic plants harboring *CpWRKY* in response to abiotic stress will provide fundamental knowledge in the agriculture industry, particularly for papaya in order to sustain its production under unfavourable environmental conditions.

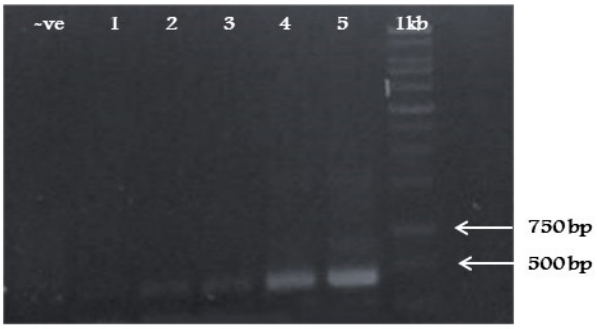

Figure 5. Colony PCR of pGEMT-*WRKY4* transformants. All colonies except No. 1 possessed the correct *WRKY* amplified fragment. Lanes 1 – 5: PCR of bacterial colonies using WRKY4 primers.

CONCLUSION

A full coding cDNA encoding WRKY has been isolated from *C. papaya* and designated as *CpWRKY4.* DNA sequencing analysis revealed that this isolated WRKY is closely related to *C.papaya* with 97% similarity. Cloning of the *CpWRKY4* into the plant expression vector pCAMBIA 1304 prior to transformation into *Arabidopsis thaliana,* for further functional studies, is currently in progress.

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