

# APPLICATION OF RNAi TECHNOLOGY FOR FUNCTIONAL ANALYSIS OF A NOVEL RIPENING-RELATED GENE

Zamri Zainal <sup>1,2</sup>, Zeti Azura Mohamed Hussein <sup>1,2</sup>, Shamsiah, A. <sup>1,3</sup> and Ismanizan Ismail<sup>1,2</sup>

<sup>1</sup>School of Bioscience and Biotechnology, UKM, Bangi

<sup>2</sup>Institute System Biology, UKM, Bangi

<sup>3</sup>Faculty of Applied Sciences, UiTM Shah Alam

## ABSTRACT

A clone designated CUKM15 was isolated from *Capsicum annum* cDNA library. Based on transcript analysis it is thought that CUKM15 is involved in fruit ripening. Searching for homologous sequences in data banks using the BLAST program revealed that CUKM15 has a putative conserve domain and showed high identity with cyclases from different organisms. Motif analysis showed that CUKM15 consists of a putative cyclase conserved domain (HXGTHXDXPXH). Nevertheless, the exact function of this gene during fruit ripening is unknown. Therefore, in this current research, we employed RNA interference (RNAi) technology to gain better understanding of its function during fruit ripening. In order to achieve the above mentioned goal, two self-complementary (termed hairpin, hp) RNA constructs with the size 456-bp and 500-bp from 5' and 3' ends were successfully produced in pHellsgate 8 and transformed in *Solanum lycopersicon*. A number of positive transformants were analysed through PCR, giving an expected band size of ~500 bp. The transcripts were successfully down regulated during the ripening and the transgenic plant harbouring CUKM15 RNAi construct exhibited significant differences in terms of its morphology by yielding smaller, irregular shaped and seedless fruit as compared to control plant.

**KEYWORDS:** *CUKM15, Capsicum annum, RNAi, cyclase*

## INTRODUCTION

Fruit ripening is a complex development process that involves changes in gene expression and enzyme activity. Fruit ripening process is of prime importance in improving fruit quality and storage potential. Isolation of transcripts encoding proteins associated with the plant development especially ripening process using differential screening or differential display techniques, has lead to discovery of novel genes with unknown function. The major challenge is to determine the functions of all the genes in the plant genomes. A straightforward approach to this problem is to reduce or knock out expression of a gene with the hope of seeing phenotype that is suggestive of its function. In this study RNA interference (RNAi) is used because of its high specificity and efficacy (Helliwell & Waterhouse, 2003). This technique is widely used as an efficient tool to analyze gene function. With the advancement of bioinformatics tools, initial protein characterization and functional can be predicted in a shortest time. Analysis through bioinformatics tools can shorten the ways of finding the function of specific gene by generating a predicted size, structure and motif of the target protein. The relationship between organisms in the phylogenetic tree will give some clues about the function of CUKM15.

## METHODOLOGY

### *In silico* analysis

Homology search was performed by using BLAST program available at NCBI. Multiple sequence alignment was done to understand the relationship between several sequences of genetic information. Existing sequence which its protein structure has been solved, was used as a template to build CUKM15 3D structure (Altschul et al., 1997).

### Recombinant plasmid construction

The fragment CUKM15-5' and CUKM15-3' were amplified using gene-specific primers modified with either a 5'-attB1 extension (5'-GGGGACAAGTTTGTACAAAAAAGC-AGGCT-3') or a attB2 extension (5'-GGGACCACTTTGTACAAGAAAGCTGGGT-3') using Tag DNA polymerase (Fermentas). Recombination reactions of PCR products with pDONR221 (Invitrogen, Groningen, The Netherlands) were carried out according to protocol provided by Invitrogen. An aliquot of the recombination reaction mix (1-2  $\mu$ L) was used to transform DH5 $\alpha$ , and colonies were selected on the kanamycin (50 mg/ml) plates. Clones were checked by PCR using M13 primer. Recombination reaction from pDONR221 clones to pHellsgate 8 were carried out in a total volume with 2  $\mu$ L pDONR221 clone (approximately 150 ng), 300 ng pHellsgate 8 and 2  $\mu$ L LR clonase enzyme mix (Invitrogen). The reaction was incubated overnight at room temperature, then treated with proteinase and used to transform DH5 $\alpha$  for the BP clonase reaction.

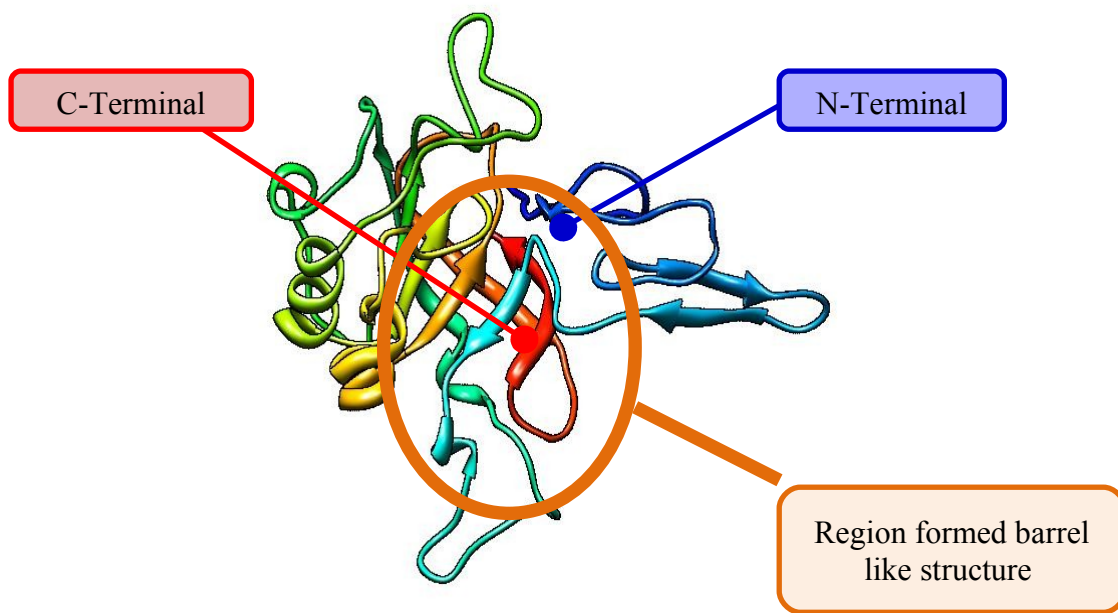
### Genetic Transformation

Seeds of *Solanum lycopersicon* Mill cultivars MT11 (obtained from MARDI) were surface sterilized in 20% (v/v) sodium hypochlorite solution for 20 min followed by three rinses in sterile distilled water and germinated aseptically on Murashige and Skog (1962) basal medium. Cotyledon and hypocotyls explants from 14-days-old seedling were isolated and cultured onto preculture media for two days preculture period. Then explant were soaked in an *Agrobacterium tumefaciens* strain LBA4404 containing culture, blotted and recultured on the same media for two days co-cultivation period. Explants were then transferred to fresh media of different plant growth regulator combinations containing 100 mgL<sup>-1</sup> kanamycin with 200 mgL<sup>-1</sup> Carbenicillin to control *Agrobacterium* growth.

## RESULTS & DISCUSSION

### *In silico* analysis

Homologous search on CUKM15 against several gene databases using the BLAST program showed that CUKM15 has the highest identity, 84% and E value at  $6.8e^{-103}$  with cyclase of *Arabidopsis thaliana*. Motif analysis showed that CUKM15 consists of a putative cyclase conserved domain located from residue 59 to 259. The conserved domain of putative cyclase is HXGTHXDXPXH. The 3-D structure of this protein was successfully constructed by differential modelling approach. The catalytic residues were present at His<sub>74</sub>, Gly<sub>78</sub>, Thr<sub>77</sub>, His<sub>78</sub>, Asp<sub>80</sub>, Pro<sub>82</sub>, His<sub>84</sub> (Figure 1).



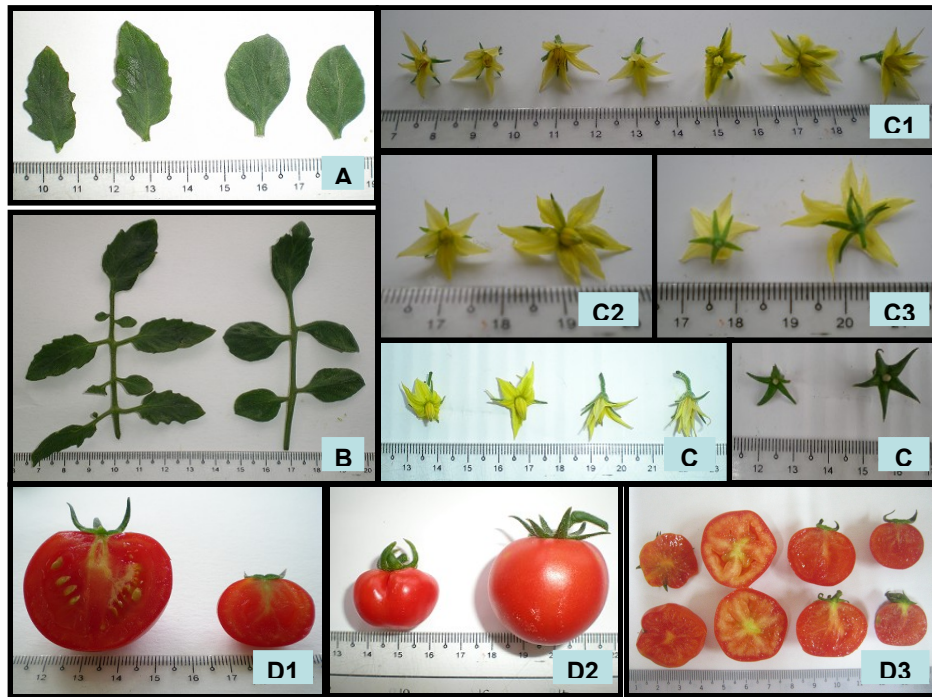
**Figure 2.** 3D model of CUKM15

### **Construction of transformation vector in pHellgate8**

The pHellgate8 was designed to accept inserts from such clones by recombination with two attR cassettes in a single step, generating an ihpRNA construct. In this recombination reaction, CUKM15 fragment was recombined into an intermediate vector, pDONR221 before recombination into pHellgate8. Only one type of recombination occurred which the intron is sense orientation with respect to the direction of transcription.

### **Genetic Transformation**

For transformation,  $\pm 4000$  cotyledons and hypocotyls were co-cultivated with *Agrobacterium* for 48 h. The callus induction was observed after 15 days of inoculation on the selection medium. At this stage almost 70% of explants survived. During the next stage, 100% of the explants were elongated and after transfer to rooting medium, these putative transgenic were transplanted on soil. The transgenic nature of these plants was confirmed by several analysis such as PCR, Southern and Northern. A specific primer derived from OCS terminator region designed to amplify a 0.4Kb fragment. The amplified DNA produced 0.4Kb fragment from transformed plants but not from non-transformed plants. Down regulating *CUKM15* expression in transgenic tomato plant has led to severe growth abnormalities in particularly reduction in size of flowers and fruits.



**Figure 3.** Morphological changes of transgenic tomato (WT= Wild type; T: Transgenic)

## CONCLUSION

In conclusion, obviously CUKM15 plays an important role in plant development. Silencing this gene has resulted in fruits with smaller sizes, irregular shape and seedless. Furthermore the leaf and flower shapes and sizes were different between wild type and transgenic. Further studies of the effect of down regulation of CUKM15 will provide some insight about its roles in the development of reproductive organs.

## ACKNOWLEDGEMENT

Ministry of Science Technology and Innovation (MOSTI) is acknowledged for providing research grant (Science Fund) to Dr. Zamri Zainal and Centre for Plant Biotechnology, INBIOSIS UKM is acknowledged for equipment and facilities support.

## REFERENCES

1. Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W., & Lipman D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25(17)**: 3389-3402.
2. Helliwell, C.A. & Waterhouse, P.M. (2003). Construct and Methodd fo high-throughput vectors for efficient gene silencing in plants. *Methods* **30**: 289-296.
3. Helliwell, C.A., Wesley, S.V., Wielopolska, A.J. & Waterhouse, P.M. (2002). High-throughput vectors for efficient gene silencing in plants. *Functional Plant Biology* **29**: 1217-1225.