UNIVERSITI PUTRA MALAYSIA

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By

ROGAYAH SEKELI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

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Abstract of this thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Janna Ong Abdullah, PhD

Faculty : Biotechnology and Biomolecular Sciences

Papaya (*Carica papaya* L.) is a popular fruit in the world. In Malaysia, among the popularly grown cultivar is Eksotika, introduced by MARDI in 1987. Similar to other tropical fruits, an Eksotika fruit has a very short shelf-life, which limits its export potential to distant destinations. Hence, there is a need to extend its shelf-life in order to reduce post-harvest losses and to increase its export potential to distant markets. This project is aimed at extending the shelf-life of the highly perishable Eksotika papaya fruit. Fruit ripening is closely related to the production of ethylene gas within the fruit. One way to extend the shelf life of papaya fruit is by manipulating the activities of the enzymes involved in ethylene biosynthesis. It was hypothesized that reduction in the production of ethylene would result in lengthening the shelf-life of the fruit. In this study, RNA interference (RNAi) and antisense RNA technologies were applied to manipulate and transform both genes encoding *1-aminocyclopropane-1-carboxylic acid* (*ACC*) *oxidase* 1 (designated as *ACO*1) and 2 (designated as *ACO*2) into Eksotika papaya embryogenic cultures. It was reported *ACO*2 is closely associated with fruit ripening characteristic compared to *ACO*1. Thus for the antisense study, only *ACO2* gene manipulation was pursued. A total of 15,000 embryogenic calli of Eksotika papaya were transformed with the three different RNAi constructs (pRNAiACO1, pRNAiACO2 and pRNAiCACO) constructs and 6,000 with the antisense *ACO*2 construct. A total of 148 positive putative transformants were recovered using the RNAi constructs, and 46 using the antisense *ACO*2 construct. Gene expression analysis using real-time RT-PCR on the antisense putative transgenic R_0 plants showed between two to five folds down- regulation of the *ACO*2 in 42 putative transgenic R_0 plants with the highest reduction shown in R_0 3-1

and R_0 27-3. For RNAi, 9 independent R_0 plants were tested and all showed between two to three folds down- regulation of the *ACO* genes. An improved and efficient rooting method was established for the regenerated putative transgenic Malaysian Eksotika papaya shoots. The rooting percentage was increased to 92.5% using the half strength Murashige & Skoog (MS) ingredients mixed with vermiculite compared to 22% using the original method comprised of the De Fossard medium. The survival rate of the rooted shoots after transfer into the ground was 92%. Morpho-histological analyses revealed that the tap roots of the shoots were more compact, which might have contributed to their high survival rates. A total of 31 independently selected RNAi plants and 24 antisense plants were transferred into soil and grown under nethouse condition for assessment of delayed ripening characteristic of the papaya fruits. Twenty RNAi and 13 antisense transgenic R_0 plants showed single copy number. Statistical analysis showed no significant difference ($p<0.05$) in plant growth performance between transgenic and non-transformed seedling-derived plants. Shelf-life analysis of the transgenic fruits showed that fruits from 11 transgenic antisense R_0 plants exhibited delayed fruit ripening with the most potential, transgenic R_0 27-3, remaining green for 14 days compared to the control (4 days). For RNAi transgenic plants, fruits from 13 R_0 plants showed delayed ripening, with the most potential R_0 plants pRNAiACO2 L2-9 and pRNAiACO1 L2 exhibited about 20 and 14 days post-harvesting to reach the full maturity index (Index 6), respectively. The total soluble solid (TSS) of the transgenic fruits was comparable to the control fruits with similar 11-14°Brix. The transgenic fruits remained firm for additional 4 to 8 days at room temperature (25 ± 2ºC) after achieving Index 6 while the non-transformed seed-derived fruits lost their firmness after 2 days. Histological studies on the transgenic and control fruits at Index 2 and Index 6 showed significant differences in their cells morphology. Overall, the findings in this study demonstrated that reduction of ethylene was successful in the Eksotika papaya by manipulating the *ACO* gene(s) using the antisense RNA and RNAi techniques. This reflects that future production of longer shelf-life Eksotika papaya fruits is possible with either antisense RNA or RNAi.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PEMBANGUNAN PEMANJANGAN TEMPOH KEMASAKAN BUAH BETIK (*Carica papaya* L.) KULTIVAR 'EKSOTIKA' DENGAN MENGGUNAKAN TEKNOLOGI RNA *INTERFERENCE* DAN *ANTISENSE*

Oleh

ROGAYAH SEKELI

Disember 2013

Pengerusi : Profesor Madya Janna Ong Abdullah, PhD

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Betik (*Carica papaya* L.) merupakan antara buah penting di dunia. Di Malaysia, antara kultivar yang popular ditanam ialah Eksotika yang diperkenalkan oleh MARDI pada tahun 1987. Sama seperti buah tropika yang lain, buah betik Eksotika mempunyai jangkahayat yang sangat singkat yang menghadkan potensi eksport ke destinasi yang lebih jauh. Oleh itu, adalah penting untuk meningkatkan jangkahayat buah untuk mengurangkan kerugian selepas tuai dan untuk meningkatkan potensi eksport ke destinasi yang lebih jauh. Projek ini bertujuan untuk memanjangkan jangkahayat buah betik Eksotika yang terlalu mudah rosak. Kemasakan buah adalah berkait rapat dengan pengeluaran gas etilena. Satu cara untuk memanjangkan jangkahayat buah betik ialah dengan mengurangkan aktiviti-aktiviti enzim yang terlibat dalam biosintesis gas etilena. Dihipotesiskan bahawa pengurangan pengeluaran gas etilena akan mengakibatkan jangkahayat buah dapat dipanjangkan. Dalam kajian ini dua teknologi yang berbeza, antisense dan RNA interference (RNAi) digunakan untuk memanipulasi dan mentransformasi kedua-dua gen yang mengkod 1-*aminocyclopropane-1-carboxyilic acid (ACC) oxidase* 1 (dinamakan sebagai *ACO*1) dan 2 (dinamakan sebagai *ACO*2) ke dalam kalus embriogenik betik Eksotika. Dilaporkan *ACO*2 lebih berkaitan dengan kemasakan buah berbanding *ACO*1. Oleh itu untuk kajian antisense, hanya *ACO*2 gen dimanipulasi. Sebanyak 15,000 kalus embryogenik telah ditransformasikan dengan ketiga-tiga konstruk RNAi (pRNAiACO1, pRNAiACO2 dan pRNAiCACO) dan 6,000 dengan konstruk *ACO*2 antisense. Sejumlah 148 positif putatif transforman telah diperolehi daripada transformasi menggunakan konstruk RNAi dan 46 daripada konstruk *ACO*2 antisense.

Analisis pengekpresan gen dengan menggunakan real-time RT-PCR ke atas pokok R_0 putatif transgenik antisense menunjukkan penurunan kadar *ACO*2 dalam 42 pokok R_0 putatif transgenik dengan kadar penurunan tertinggi ditunjukkan oleh R_0 3-1 dan R_0 27-3. Untuk RNAi, 9 pokok R_0 berlainan telah diuji dan kesemuanya menunjukkan kadar penurunan 2-3 kali ganda gene *ACO*. Kaedah pengakaran yang lebih baik dan cekap telah berjaya dibangunkan untuk regenerasi pucuk betik Eksotika Malaysia yang putatif transgenik. Peratusan pengakaran telah meningkat kepada 92.5% dengan menggunakan setengah kepekatan Murashige & Skoog (MS) yang dicampur dengan vermiculite berbanding 22% menggunakan kaedah asal yang mengandungi media De Fossard. Kadar kemandirian pucuk selepas pemindahan ke tanah ialah 92%. Analisis morpho-histologi menunjukkan bahawa akar tunjang adalah lebih padat yang mungkin telah menyumbang kepada kadar kemandirian yang tinggi. Sebanyak 31 pokok RNAi dan 24 pokok antisense yang dipilih secara rawak telah dipindahkan ke tanah dan ditanam di bawah rumah jaring untuk penilaian ciri pemanjangan tempoh kemasakan buah betik. Dua puluh pokok R_0 transgenik RNAi dan 13 pokok R_0 transgenik antisense menunjukkan bilangan salinan tunggal. Analisis statistik menunjukkan tiada perbezaan yang signifikan antara pokok transgenik dan pokok kawalan. Analisis jangkahayat menunjukkan 11 pokok R_0 transgenik antisense mempamerkan kemasakan buah yang lambat dengan pencapaian terbaik iaitu transgenik pokok R_0 27-3 yang kekal hijau selama 14 hari berbanding dengan pokok kawalan (4 hari). Bagi pokok transgenik RNAi, 13 pokok R_0 menunjukkan kemasakan buah yang lambat dengan barisan yang paling berpotensi iaitu pRNAiACO2 R_0 2-9 dan pRNAiACO1 R_0 2 yang mengambil masa kira-kira 20 dan 14 hari selepas dituai untuk mencapai kemasakan penuh (Indek 6). Perbandingan jumlah pepejal larut di antara buah transgenik dan buah kawalan menunjukkan profil yang serupa, 11-14 °Brix. Buah transgenik masih tetap kukuh untuk 4-8 hari pada suhu bilik (25 ± 2℃) selepas mencapai Indek 6, manakala buah kawalan menjadi lembut hanya selepas 2 hari. Analisis histologi menunjukkan perbezaan sel morfologi antara buah transgenik dan buah kawalan pada Indek 2 dan 6. Hasil kajian menunjukkan bahawa pengeluaran gas etilena berjaya diturunkan melalui manipulasi gen *ACO* ke dalam betik Eksotika dengan menggunakan kedua-dua teknik antisense dan RNAi. Ini mengambarkan bahawa penghasilan betik Eksotika yang mempunyai jangkahayat buah yang panjang boleh didapati samada dengan teknik antisense ataupun RNAi.

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I certify that a Thesis Examination Committee has met on 13 December 2013 to conduct the final examination of Rogayah binti Sekeli on her thesis entitled "Development of Delayed Ripening Papaya (*Carica papaya* L.) CV. 'Eksotika' using RNA Interference and Antisense Technologies" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

ROGAYAH BINTI SEKELI

Date: 13 December 2013

TABLE OF CONTENTS