
**UNIVERSITI PUTRA MALAYSIA**

# DEVELOPMENT OF PLANT REGENERATION SYSTEM AND *AGROBACTERIUM*- MEDIATED TRANSFORMATION OF *BRASSICA OLERACEA* L. SUBSP. *ITALICA* cv. GREEN MARVEL WITH HSP101 GENE

**SEYED ALI RAVANFAR**

**FP 2012 49**

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**By**

**SEYED ALI RAVANFAR**



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

**December 2012**

*In The Name of ALLAH, the Most Gracious and the Most Merciful*

*Specially Dedicated*

*To*

*My beloved wife Shaghayegh*

*My parents Seyed Naser and Zarintaj*

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

**DEVELOPMENT OF PLANT REGENERATION SYSTEM AND *AGROBACTERIUM*- MEDIATED TRANSFORMATION OF *BRASSICA OLERACEA* L. SUBSP. *ITALICA* cv. GREEN MARVEL WITH HSP101 GENE**

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**Chairman : Associate Professor Maheran Bt Abd. Aziz, PhD**

**Faculty : Agriculture**

Broccoli seeds are among the most commonly imported vegetable seeds in Malaysia. In Malaysia due to the humid climate, production of hybrid seeds is almost impossible. Consequently, improvement of *in vitro* culture method for clonal propagation of broccoli plants having the $F_1$ hybrid characteristics is essential. Broccoli plants respond adversely to extreme temperatures and high humidity in the lowland thus, gene transformation for heat tolerance would be beneficial. Cameron Highlands is the main broccoli producing area in Malaysia because of the suitable cool climate and the cultivar that is commonly grown is Green Marvel. Therefore, the main objectives of this study were to improve the shoot regeneration system for *Brassica oleracea* subsp. *italica* cv. Green Marvel and to introduce *Arabidopsis thaliana* HSP101 (*At*hsp101) cDNA into broccoli through *Agrobacterium tumefaciens*-mediated transformation in order to

increase its heat-tolerance characteristic. Multiple shoot formation from hypocotyl and shoot tip explants were assessed using different concentrations of TDZ (thidiazuron), zeatin and kinetin. In the experiment on multiple shoot formation on hypocotyl explants, TDZ at 0.1 mg/l induced the highest percentage of explant with shoot (96.67%) and the highest mean number of shoots per explant (6.17). In the experiment on shoot multiplication from shoot tip explants, the highest percentage of shoot tip explant producing shoots (100%) was on medium with 0.1 mg/l TDZ followed 1.5 mg/l zeatin (96.67%), while, the highest number of shoots per explant (4.27) was on 1.5 mg/l zeatin. Therefore, 0.1 mg/l TDZ was considered the most suitable for adventitious shoot formation from hypocotyl explants and 1.5 mg/l zeatin from shoot tip. In the determination of minimum inhibitory concentration (MIC) of kanamycin for effective screening of broccoli transformants, the lowest percentage (0.0%) and mean number of survived hypocotyl explants (0.00) was on shoot regeneration medium (SRM) containing 60 mg/l kanamaycin, while the lowest percentage (0.0%) and mean number of survived shoot tip explants (0.00) occurred on SRM containing 90 and 100 mg/l kanamycin. Therefore, 50 mg/l and 80 mg/l kanamaycin were the chosen MIC for hypocotyl and shoot tip explants, respectively. In the optimization of factors affecting *Agrobacterium* mediated-transformation of broccoli with *At*HSP101 gene and the regeneration of putative transformed plantlets, hypocotyls explants precultured on SRM with 200µM acetosyringone produced the highest percentage (13.33 %) and mean number of putative transformants (0.17), while shoot tip explants precultured on callus induction medium (CIM) with 200µM acetosyringone produced the highest

percentage (23.33%) and mean number of putative transformants (0.27) after 8 weeks of culture. Optimization of bacterial dilution and inoculation time showed that the inoculation of hypocotyl segments in 1:5 bacterial dilution for 30 min produced the highest percentage (20 %) and mean number (0.27) of putative transformants. The same bacterial dilution and inoculation time also produced the highest percentage (30%) and mean number (0.33) of putative transformant from shoot tip explants. Thus, preculture with 200μM acetosyringone followed by inoculation in (1:5) bacterial dilution for 30 min was the most successful for transformation of broccoli with *At*HSP101 gene. PCR analysis showed the expected fragment size of the *At*HSP101 gene, while Southern blot analysis showed different hybridization bands in the hypocotyl (1 and 2 gene copy number) and shoot tip (3 gene copy number) derived transformants. The gene expression was confirmed through reverse transcriptase (RT-PCR) assay. Consequently, the transgenic broccoli plantlets were transferred to different temperature regimes (20ºC, 30ºC and 34ºC) in the transgenic greenhouse to evaluate the efficacy of HSP 101 gene in increasing their heat tolerance. Results showed that the transgenic plants could survive and performed normally, producing flower heads even at the highest tested temperature of 34ºC. In conclusion, an improved regeneration system has been established from hypocotyl and shoot tip explants of broccoli followed by successful transformation with *At*HSP101 for resistance to high temperature.

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

**PEMBENGUNAN SISTEM REGENERASI DAN TRANSFORMASI *BRASSICA OLERACEA* L. SUBSP. *ITALICA* cv. GREEN MARVEL DENGAN HSP101 GEN MELALUI PENGANTARAAN *AGROBACTERIUM***

Oleh

**SEYED ALI RAVANFAR**

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Benih brokoli adalah di antara benih sayur-sayuran yang paling biasa diimport di Malaysia. Oleh kerana iklimnya yang panas dan lembap, pengeluaran biji benih hibrid adalah hampir mustahil di Malaysia. Oleh itu, pembaikan kaedah *in vitro* untuk pembiakan klonal tumbuhan brokoli yang mempunyai ciri-ciri hibrid $F_1$ adalah diperlukan. Tumbuhan brokoli bertindak balas sebaliknya kepada suhu yang melampau dan kelembapan yang tinggi di tanah rendah, maka transformasi gen untuk toleransi terhadap haba adalah bermanafaat. Cameron Highlands, yang beriklim sederhana sejuk, adalah kawasan pengeluaran brokoli yang utama di Malaysia dan kultivar yang biasanya ditanam ialah Green Marvel. Oleh itu, objektif utama kajian ini adalah untuk memperbaiki sistem regenerasi pucuk untuk *Brassica oleracea* subsp. *italica* cv. Green Marvel dan untuk memasukkan HSP101 (*At*hsp101) cDNA daripada *Arabidopsis thaliana* ke

dalam brokoli melalui transformasi dengan pengantaraan *Agrobacterium tumefaciens* untuk meningkatkan ciri toleransi brokoli terhadap haba. Pembentukan pucuk berganda daripada eksplan hipokotil dan hujung pucuk dikaji menggunakan kepekatan thidiazuron (TDZ), zeatin dan kinetin yang berbeza. TDZ pada 0.1 mg/l menjana peratusan tertinggi pengeluaran pucuk (96,67%) dan bilangan pucuk tertinggi (6,17) daripada eksplan hipokotil. Sementara, peratusan tertinggi eksplan hujung pucuk menghasilkan pucuk (100%) adalah pada medium mengandungi 0.1 mg/l TDZ diikuti 1.5 mg/l zeatin (96,67), manakala, jumlah tertinggi pucuk per eksplan (4.27) pada 1.5 mg/l zeatin. Maka 0.1 mg/l TDZ adalah dianggap paling sesuai untuk pembentukan pucuk daripada eksplan hypokotil dan 1.5 mg/l zeatin daripada eksplan pucuk. Untuk saringan transforman brokoli yang berkesan, 50 mg/l dan 80 mg/l kanamisin dipilih sebagai kepekatan perencatan minimum (MIC) kanamisin, masing-masing bagi eksplan hipokotil dan pucuk. Bagi pengoptimuman faktor yang mempengaruhi transformasi brokoli dengan gen *At*HSP101 berperantaraan-*Agrobacterium* dan regenerasi transforman putatif, eksplan hipokotil yang diprakultur di atas SRM dengan 200µM asetosringon menghasilkan peratusan (13.33%) dan min bilangan transforman putatif (0.17) tertinggi, manakala eksplan hujung pucuk yang diprakultur di atas medium induksi kalus (CIM) dengan 200µM asetosringon menghasilkan peratusan (23.33%) dan min bilangan transforman (0.27) tertinggi selepas 8 minggu dikultur. Pengoptimuman pencairan bakteria dan masa inokulasi menunjukkan bahawa inokulasi segmen hipokotil dalam pencairan bakteria (1:5) selama 30 minit menghasilkan peratusan (20%) dan min bilangan transforman (0.27)

tertinggi. Pencairan bakteria dan masa inokulasi yang sama juga menghasilkan peratusan (30%) dan min bilangan transforman (0.33) tertinggi daripada eksplan hujung pucuk. Oleh itu, prakultur dengan asetosringon 200μM yang diikuti dengan inokulasi dalam pencairan bacteria (1:5) selama 30 minit adalah yang paling berjaya untuk transformasi brokoli dengan gen *At*HSP101. Analysis PCR menunjukkan serpihan saiz jangkaan gen *At*HSP101, sementara analisis penyerapan Southern menunjukkan band penghibridan yang berbeza bagi transforman yang diperolehi daripada eksplan hipokotil (1 dan 2 bilangan salinan gen) dan hujung pucuk (3 bilangan salinan gen). Pengekspresan gen telah disahkan melalui asai transkriptas berbalik (RT-PCR). Seterusnya, anak pokok brokoli transgenik telah dipindahkan ke dalam rejim suhu yang berbeza (20ºC, 30ºC dan 34ºC) dalam rumah hijau transgenik untuk menilai keberkesanan gen *At*HSP 101 dalam meningkatkan toleransi pokok tersebut terhadap haba. Keputusan menunjukkan bahawa tumbuhan transgenik boleh hidup normal dan menghasilkan kepala bunga walaupun pada suhu tertinggi yang diuji iaitu 34 ºC. Rumusannya, sistem regenerasi yang diperbaiki telah dibangunkan daripada eksplan hipokotil dan hujung pucuk brokoli diikuti dengan kejayaan transformasi dengan gen *At*HSP101 untuk toleran terhadap suhu tinggi.

## ACKNOWLEDGEMENTS

### IN THE NAME OF ALLAH

Thanks and Praise are due to Allah who gave me strength and determination to complete my study. I would like to express my gratitude and sincere thanks to those who have helped me in preparing and conducting the research and finishing this thesis. Therefore, it pleases me to express my deep gratitude to them.

I would like to thank the chairman of my supervisory committee, Associate Professor Dr. Maheran Abdul Aziz, for her supervision, kind guidance and advice in the completion of the thesis. I appreciate her patience and sincere approach to motivate, help, advice and guide me to finish my study. Thanks are also extended to my committee members, Associate Professor Dr. Halimi bin Mohd Saud and Associate Professor Dr. Janna Ong Abdullah. The experience and knowledge, gained has resulted in a significant and positive effect on my work.

Last but not least, thanks to my forever love Shaghayegh, my father Naser, my mother Zarin my father and mother in law Mehrdad and Mina, my brothers Roham and Mohammad. I owe you everything.

I certify that a Thesis Examination Committee has met on 31 December 2012 to conduct the final examination of Seyed Ali Ravanfar on his thesis entitled "Development of Plant Regeneration System and *Agrobacterium*-Mediated Transformation of *Brassica oleracea* subsp. *italica* cv. Green Marvel with HSP101 Gene" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1988. The Committee recommends that the student be awarded the Doctor of Philosophy.
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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment 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 for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and or not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

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**Seyed Ali Ravanfar**

Date: 31 December 2012

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