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

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 All\u0627\u0646, the Most Gracious and the Most Merciful

Specially Dedicated

To

My beloved wife Shaghayegh

My parents Sayed 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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December 2012

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 F₁ 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 (AtHsp101) 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 (0.0) was on shoot regeneration medium (SRM) containing 60 mg/l kanamycin, while the lowest percentage (0.0%) and mean number of survived shoot tip explants (0.0) occurred on SRM containing 90 and 100 mg/l kanamycin. Therefore, 50 mg/l and 80 mg/l kanamycin were the chosen MIC for hypocotyl and shoot tip explants, respectively. In the optimization of factors affecting Agrobacterium mediated-transformation of broccoli with AtHSP101 gene and the regeneration of putative transformed plantlets, hypocotyls explants precultured on SRM with 200μM acertosyringone 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 acertosyringone 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 AtHSP101 gene. PCR analysis showed the expected fragment size of the AtHSP101 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 AtHSP101 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
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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 hipokotil 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 *AtHSP101* 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 AtHSP101. Analysis PCR menunjukkan serpihan saiz jangkaan gen AtHSP101, 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 AtHSP 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 AtHSP101 untuk toleran terhadap suhu tinggi.
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IN THE NAME OF ALLAH

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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. Members of the Thesis Examination Committee were as follows:

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Date:
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.

__________________
Seyed Ali Ravanfar

Date: 31 December 2012
# TABLE OF CONTENTS

## DEDICATION

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ii</td>
</tr>
</tbody>
</table>

## ABSTRACT

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>iii</td>
</tr>
</tbody>
</table>

## ABSTRAK

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>vi</td>
</tr>
</tbody>
</table>

## ACKNOWLEDGEMENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ix</td>
</tr>
</tbody>
</table>

## APPROVAL

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
</tr>
</tbody>
</table>

## DECLARATION

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>xii</td>
</tr>
</tbody>
</table>

## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>xvii</td>
</tr>
</tbody>
</table>

## LIST OF TABLES

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>xxi</td>
</tr>
</tbody>
</table>

## LIST OF PLATES

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>xxiii</td>
</tr>
</tbody>
</table>

## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>xxiv</td>
</tr>
</tbody>
</table>

## CHAPTER

### 1 INTRODUCTION

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

### 2 LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

2.1 Origin, Taxonomy and Botany of Broccoli 5

2.2 *In vitro* Plant Regeneration of *Brassicas* 7

2.3 High Temperature Stress (HTS) 9

2.3.1 High Temperature Stress in Broccoli 9

2.3.2 Heat Shock Protein 10

2.4 *Agrobacterium*-Mediated Transformation 12

2.4.1 *Agrobacterium* 12

2.4.2 Plant Transformation Binary Vector 14

2.4.2.1 pGreen Binary Vector 15

2.4.3 Selectable Marker Genes 16

2.4.4 Reporter Marker Gene 17

2.4.5 Preculture and Transformation Enhancers 19

2.5 *Agrobacterium Tumefaciens*-Mediated Transformation 21

2.5.1 *Agrobacterium Tumefaciens* -Mediated of *Brassicas* 22

2.5.2 Luciferase Assay 26

2.5.3 Reverse transcriptase (RT-PCR) 27

2.5.4 Southern Blotting 28

2.6 Acclimatisation of Transgenic plant 29
3 SHOOT REGENERATION FROM HYPOCHOTYL AND SHOOT TIP EXPLANTS USING DIFFERENT CONCENTRATIONS OF TDZ, ZEATIN AND KINETIN

3.1 Introduction
3.2 Materials and Methods
  3.2.1 Seed Sterilization and Germination Procedure
  3.2.2 Explant Preparation and Culture
  3.2.3 Basic Medium
  3.2.4 Experiments
  3.2.5 Experimental Design and Statistical Analysis
3.3 Results
  3.3.1 Effect of Different Concentrations of TDZ, Zeatin and Kinetin on Percentage and Mean Number of Shoot Formation from Hypocotyl Explants
  3.3.2 Effect of Different Concentrations of TDZ, Zeatin and Kinetin on Percentage and Mean Number of Shoot Formation from Shoot tip Explants
  3.3.3 Comparison of the Best Concentration of TDZ, Zeatin and Kinetin on Percentage of Shoot Formation and Mean Number of Shoots Produced Per Hypocotyl and Shoot Tip Explant of Broccoli cv. Green Marvel
3.4 Discussion
3.5 Conclusion

4 DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF KANAMYCIN FOR EFFECTIVE SCREENING OTRANSGENIC BROCCOLI PLANTLETS

4.1 Introduction
4.2 Materials and Methods
  4.2.1 Seed Surface Sterilization and Plant Materials
  4.2.2 Experiments
  4.2.3 Parameters Recorded
  4.2.4 Experimental Design and Statistical Analysis
4.3 Results
  4.3.1 Determination Of Minimum Inhibitory Concentration (MIC) Of Kanamycin For Effective Screening For Hypocotyl Explants Of Broccoli cv. Green Marvel
  4.3.2 Determination of Minimum Inhibitory Concentration (MIC) of Kanamycin for Effective Screening for Shoot tip Explants of Broccoli Transformants
4.4 Discussion
4.5 Conclusion
# OPTIMIZATION OF FACTORS AFFECTING *AGROBACTERIUM* MEDIATED-TRANSFORMATION OF BROCCOLI WITH ATHSP101 GENE AND THE REGENERATION OF PUTATIVE TRANSFORMED PLANTLETS

5.1 Introduction 65

5.2 Materials and Methods 67

5.2.1 Binary vector 67

5.2.2 *Agrobacterium* 67

5.2.3 Plant Material 68

5.2.4 To Determine the Effect of Preculture and Acetosyringone on Improvement of Transformation Frequency 68

5.2.5 To Determine the Effect of Bacterial Dilution And Inoculation Time on Improvement of Transformation Frequency 72

5.3 Results 76

5.3.1 Effect of Preculture and Acetosyringone on Improvement of Transformation Frequency 76

5.3.2 Effect of Bacterial Dilution and Inoculation Time on Improvement of Transformation Frequency 81

5.4 Discussion 86

5.4.1 Effect of Preculture and Acetosyringone on Improvement of Transformation Frequency 86

5.4.2 Effect of Bacterial Dilution and Inoculation Time on Improvement of Transformation Frequency 88

5.5 Conclusion 90

# CONFIRMATION OF TRANSGENICITY OF BROCCOLI PLANTLETS AND EVALUATION ON THE EFFICACY OF HSP 101 GENE IN INCREASING THE HEAT TOLERANCE

6.1 Introduction 91

6.2 Materials and Methods 93

6.2.1 PCR Amplification 93

6.2.2 Luciferase Assay 94

6.2.3 Southern Blot Analysis 95

6.2.3.1 Broccoli Genomic DNA Extraction 95

6.2.3.2 Southern Blotting 97

6.2.4 RT-PCR 99

6.2.5 Acclimatization of Transgenic Plantlets 100

6.2.5.1 Experiment 100

6.2.5.2 Experimental Design and Statistical Analysis 101
6.3 Results
6.3.1 Polymerase Chain Reaction Analysis
6.3.2 Quantitative Luciferase Activity
6.3.3 Southern Blot Analysis
6.3.4 RT-PCR
6.3.5 Performance of Transgenic Broccoli Plantlets in the Transgenic Greenhouse

6.4 Discussion
6.5 Conclusion

7 SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

REFERENCES/BIBLIOGRAPHY
APPENDICES
BIODATA OF STUDENT
LIST OF PUBLICATIONS