UNIVERSITI PUTRA MALAYSIA

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF CABBAGE (*BRASSICA OLERACEA* SUBSP. *CAPITATA*) CV. KY CROSS WITH *AT*HSP101 GENE

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FP 2008 3

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By

ARASH RAFAT

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

March 2008

In The Name of Allah, the Most Gracious, the Most Merciful

Specially dedicated to:

My kind parents
Nader and Masoumeh

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF CABBAGE (_BRASSICA OLERACEA_ SUBSP. _CAPITATA_) CV. KY CROSS WITH _AT_HSP101 GENE

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ARASH RAFAT

December 2007

Chairperson: Associate Professor Maheran Abdul Aziz, PhD

Faculty: Agriculture

An *Agrobacterium tumefaciens*-mediated transformation was developed for cabbage (*Brassica oleracea* subsp. *capitata*) cultivar KY Cross by introduction of the *At*HSP101 gene.

The *At*HSP101 cDNA was cloned in place of gusA in pCAMBIA1301 binary vector to produce an effective binary vector construct of *At*HSP101 gene for transformation work. The construct named pCAMHSP and the successful cloning was confirmed by both restriction enzymes analysis and Polymerase Chain Reaction (PCR) assay. Electrophoresis of double digestion product of pCAMHSP binary vector (~12.5Kb) showed both the expected band sizes of ~9800 bp and

~2750 bp. Also, PCR analysis produced a ~2750 bp band which indicated the presence of the *At*HSP101 inside the pCAMHSP designed binary vector.

Before transformation, the minimum inhibitory concentration of hygromycin was determined for hypocotyl and shoot tip explants of cabbage cv. KY Cross to be used in the selection of putative transformants. Hygromycin B at 3.5 and 10 mg/L were selected as the minimum inhibitory concentrations for causing the death of hypocotyl and shoot tip explants respectively.

Two different *Agrobacterium tumefaciens* strains (C58 and GV2260) were co-cultivated with the two types of explants (hypocotyl and shoot tip) to optimize factors that could contribute to the improvement of the *Agrobacterium tumefaciens*-mediated transformation of cabbage cv. KY Cross.

The effect of preculture and acetosyringone on improvement of transformation frequency was determined by using three preculture treatments (without preculturing, preculturing on Callus Inducing Medium, and preculturing on Shoot Regeneration Medium) in combination with two different acetosyringone concentrations (0 and 100 μM)). The duration of three days preculture on the Callus Induction Medium in combination with 100 μM acetosyringone applied in

the *Agrobacterium* culture medium resulted the highest percentage of transformation from hypocotyl explants (15% and 30% using *A. tumefaciens* strain C58 and GV2260 respectively). Meanwhile, the highest percentages of transformation from shoot tip explants (12.5% and 17.5% using *A. tumefaciens* strain C58 and GV2260 respectively) were observed by the application of 100 µM acetosyringone in the *Agrobacterium* culture medium but without preculturing.

The effect of bacterial dilution and inoculation time on improvement of transformation frequency was also determined. Three different bacterial dilution rates (1:5, 1:10, and 1:15) and three different inoculation times (5, 10, and 20 min) were assessed. It was found that 1:10 *Agrobacterial* dilution and 5 minutes inoculation period resulted in the highest transformation frequency in both hypocotyl (20% and 35% using *A. tumefaciens* C58 and GV2260 respectively) and shoot tip explants (15% and 17.5% using *A. tumefaciens* C58 and GV2260 respectively).

A shoot induction system after cocultivation was established. Multiple shoot formation from hypocotyls and shoot tip explants of cabbage (*Brassica olarecea* subsp. *capitata*) cultivar KY Cross was evaluated after cocultivation on MS medium containing 500 mg/L carbenicillin and different concentrations of BAP (0, 1, 2, 3 and 5 mg/L). The highest percentage of shoot formation and mean

number of shoots for both hypocotyl (87.5% and 90% using *A. tumefaciens* C58 and GV2260 respectively) and shoot tip explants (92.5% and 95% using *A. tumefaciens* C58 and GV2260 respectively) were obtained on 2 mg/L BAP. The highest mean number of shoots for both hypocotyl (5.60 using both *A. tumefaciens* C58 and GV2260) and shoot tip explants (3.0 and 3.17 using *A. tumefaciens* C58 and GV2260 respectively) were also obtained on 2 mg/L BAP.

Confirmation of the transformed lines was determined by PCR using primers of *At*HSP101 gene. Observation of the expected sized fragment of 196 bp in some putative transformant lines confirmed the integration of *At*HSP101 gene into the transformed plants genome. The highest percentages of transformation from hypocotyl and shoot tip explants were 45% and 32.5% respectively.

Abstrak tesis dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains

TRANSFORMASI MELALUI PERANTARAAN *AGROBAKTERIUM* BAGI KOBIS (*BRASSICA OLERACEA* SUBSP. *CAPITATA*) KV. KY CROSS DENGAN MEMASUKKAN GEN *AT*HSP101

Oleh

ARASH RAFAT

December 2007

Pengerusi : Profesor Madya Maheran Abdul Aziz, PhD

Fakulti : Pertanian

Satu kaedah transformasi melalui perantaraan *Agrobacterium* telah dibangunkan bagi kobis (*Brasssica oleracea* subsp. *capitata*) kultivar KY Cross dengan memasukkan gen *At*HSP101.

cDNA daripada *At*HSP101 telah diklonkan pada tempat gusA pada vektor binari pCAMBIA1301 untuk menghasilkan satu binaan vektor binari bagi gen *At*HSP101 yang berkesan untuk transformasi. Binaan tersebut dinamakan

pCAMHSP dan kejayaan pengklonan telah disahkan dengan analisis enzim penyekat dan ujian Tindakbalas Polimerase Berantai (PCR). Elektroforesis daripada hasil pencernaan gandadua vektor binari pCAMHSP (~12.5Kb) menunjukkan saiz jalur yang telahpun dijangka iaitu ~9800bp dan ~2750bp. Juga, analisis PCR telah menghasilkan suatu jalur ~2750 bp yang menunjukkan kehadiran *At*HSP101 di dalam vektor binari pCAMHSP yang telah direkabentuk.

Sebelum transformasi, kepekatan perencatan minima higromisin telah ditentukan untuk eksplan hipokotil dan tunas pucuk kobis kv. KY Cross untuk digunakan dalam pemilihan transforman putatif. Higromisin B pada paras 3.5 dan 10 mg/L telah dipilih sebagai kepekatan perencatan minima yang menyebabkan kematian masing-masing pada eksplan hipokotil dan tunas pucuk.

Dua strain *Agrobacterium tumefaciens* yang berbeza (C58 dan GV2260) dikultur bersama dengan dua jenis eksplan (hipokotil dan tunas pucuk) untuk mengoptimakan faktor yang dapat menyumbang kepada peningkatan transformasi kobis kv. KY Cross melalui perantaraan *Agrobacterium tumefaciens*.

Kesan daripada prakultur dan asetosiringon ke atas peningkatan kekerapan transformasi telah ditentukan dengan menggunakan tiga rawatan prakultur (tanpa

prakultur, prakultur ke atas Medium Induksi Kalus, dan prakultur ke atas Medium Regenerasi Tunas) yang dikombinasikan dengan dua kepekatan asetosiringon yang berbeza (0 dan 100 μM). Prakultur selama tiga hari di atas Medium Induksi Kalus yang dikombinasikan dengan pemberian 100μM asetosiringon telah meningkatkan kekerapan transformasi pada eksplan hipokotil (masing-masing 15% pada *A. tumefaciens* strain C58 dan 30% pada GV2260). Manakala, peratusan transformasi paling tinggi daripada eksplan tunas pucuk (masing-masing 12.5% pada *A. tumefaciens* strain C58 dan 17.5% pada GV2260) telah diamati dengan penggunaan 100 μM asetosiringon dalam media kultun *Agrobacterium* tetapi tanpa prakultur.

Kesan daripada pencairan bakteria dan masa inokulasi ke atas peningkatan kekerapan transformasi telah juga ditentukan. Tiga purata pencairan bakteria yang berbeza (1:5, 1:10 dan 1:15) yang dikombinasikan dengan tiga masa inokulasi yang berbeza (5, 10 dan 20 minit) telah diuji. Didapati bahawa pencairan *Agrobacteria* 1:10 dengan masa inokulasi 5 minit menghasilkan kekerapan transformasi paling tinggi pada kedua-dua eksplan hipokotil (masing-masing 20% dengan menggunakan *A. tumefaciens* strain C58 dan 35% pada GV2260) dan tunas pucuk (masing-masing 15% dengan menggunakan *A. tumefaciens* strain C58 dan 17.5% pada GV2260).

Suatu kaedah induksi tunas setelah pengkulturan bersama telah dipastikan. Pembentukan tunas berganda daripada eksplan hipokotil dan tunas pucuk kobis (*Brassica oleracea* subsp. *capitata*) kultivar KY Cross telah dikaji setelah pengkulturan bersama di atas medium MS yang mengandungi 500 mg/L karbenisilin dan kepekatan BAP yang berbeza (0, 1, 2, 3 dan 5 mg/L). Peratus paling tinggi pembentukan tunas pada kedua-dua eksplan hipokotil (masing-masing 87.5% dengan menggunakan *A. tumefaciens* strain C58 dan 90% pada GV2260) dan tunas pucuk (masing-masing 92.5% dengan menggunakan *A. tumefaciens* strain C58 dan 95% pada GV2260) telah diperolehi pada 2 mg/L BAP. Peratus paling tinggi purata bilangan tunas pada kedua-dua eksplan hipokotil (5.60 dengan menggunakan kedua-dua *A. tumefaciens* strain C58 dan GV2260) dan eksplan tunas pucuk (3.0 dengan menggunakan *A. tumefaciens* strain C58 dan 3.17 pada GV2260) juga telah diperolehi pada 2 mg/L BAP.

Pengesahan jalur transformasi telah ditentukan melalui PCR menggunakan primer gen *At*HSP101. Pengamatan saiz serpihan 196 bp pada transforman putatif telah mengesahkan integrasi gen *At*HSP ke dalam genom tanaman yang telah ditransformasi. Peratus transformasi paling yang tinggi daripada eksplan hipokotil dan tunas pucuk masing-masing adalah 45% dan 32.5%.

ACKNOWLEDGEMENTS

In the name of Allah the compassionate the merciful, who made it possible for me to complete another step of my life, and the best regards from Allah to the last prophet, Mohammad and his family.

I would like to express my deepest gratitude to my Supervisory Committee Chairman, Associate Professor Dr. Maheran Abdul Aziz from the Department of Agriculture Technology, Faculty of Agriculture, for her guidance, constant encouragement, valuable advices and the freedom of work she provided all throughout my research.

I am thankful to Mr. Azmi Abdul Rashid (M.Phil.) and Associate Professor Dr. Siti Nor Akmar Abdullah, members of my Supervisory Committee, also from the Department of Agriculture Technology, Faculty of Agriculture. Mr. Azmi helped me not only through his support, valuable suggestions, patience in listening, and useful discussions during my research but also by his friendliness out of the research area.

I am also grateful to Prof. Elizabeth Vierling from University of Arizona USA for kindly providing the *At*HSP101 cDNA, Dr. Huseyin Anvi Oktem from Middle East Technical University of Turkey for kindly providing the *Agrobacterium tumefaciens* strain GV2260.

It is my pleasure to offer my thanks to all my laboratory mates and colleagues especially Hossein Kamaledini, Fatemeh, Ramtin, Suleiman, Syaiful, Beverlien, Dalila, Aini, Tajul, Norwaty, Chia, Chin, Vahid, Azadeh and Motahareh for maintaining a pleasant research atmosphere.

My special thanks are also due to Amir Izadfard, Hossein Torabi, Mohammad Bagher Javadi and Fazilia, my close and special friends in Malaysia who made my stay in Malaysia an enjoyable one.

I am also personally thankful to all those known and unknown faces who directly or indirectly helped me during the phase of work towards my thesis dissertation.

Last but not the least, it is difficult to word my gratitude towards my family members for their encouragement and support during this period. I am grateful to my parents and my brother for supporting me in every possible ways.

I certify that an Examination Committee has met on 31st March 2008 To conduct the final examination of Arash Rafat on his Master of Science thesis entitled "*Agrobacterium Tumefaciens*-Mediated Transformation of Cabbage (*Brassica Oleracea* Subsp. *Capitata*) cv. KY Cross with *Athsp101* Gene" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Master Science.

Member of the 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 fulfilment of the requirement for the degree of Master of Science. The members of 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 is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

ARASH RAFAT
Date: 28 April 2008

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