UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF HEAT TOLERANT STRAWBERRY (FRAGARIA X ANANASSA DUCH., cv. CAMAROSA) PLANTS THROUGH AGROBACTERIUM-MEDIATED TRANSFORMATION

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DEVELOPMENT OF HEAT TOLERANT STRAWBERRY (*FRAGARIA X ANANASSA* DUCH., cv. CAMAROSA) PLANTS THROUGH *AGROBACTERIUM*-MEDIATED TRANSFORMATION

By

FATEMEH HADDADI

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

December 2012

In the name of ALLAH

Specially Dedicated to

All love

Specially

Hossein

My parents Habib and Parvin

And my brothers Farshad and Farzad

For their loving support

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

DEVELOPMENT OF HEAT TOLERANT STRAWBERRY (*FRAGARIA X ANANASSA* DUCH., cv. CAMAROSA) PLANTS THROUGH *AGROBACTERIUM*-MEDIATED TRANSFORMATION

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In Malaysia strawberry production is limited to the highland areas. Hence, based on the limitation of production areas the application of genetic engineering to produce a heat tolerant strawberry may facilitate the cultivation of the crop on the lowland areas. An *Agrobacterium*-mediated transformation method was applied to introduce the *At*HSP101 cDNA into strawberry cv. Camarosa. The *At*HSP101 cDNA under the control of CaMV35S promoter was cloned into the multiple cloning sites of pGreen0049. The construct was named pGFHSP and successfully introduced into *Agrobacterium* LBA4404. *In vitro* regeneration system from strawberry leaves used in the transformation was optimized by the application of different thidiazuron (TDZ) concentrations in MS medium. TDZ at 16 µM showed the highest percentage (100 %) of shoot formation and the highest mean number of shoots (24) produced per explant. Effect of different antibiotics (timentin, cefotaxime, carbenicillin and ampicillin) on shoot regeneration of strawberry leaf explants showed the best shoot regeneration in the presence of 300 mg/l timentin and 150 mg/l cefotaxime. Determination of the minimum inhibitory concentration (MIC) of kanamycin was

carried out using leaf explant for effective screening of strawberry putative transformants. Kanamycin at 50 mg/l, with shoot regeneration percentage of 0 % and mean number of 0 shoot per explant was selected as the MIC. Assessment of different factors affecting *Agrobacterium* mediated-transformation of strawberry with the *At*HSP101 showed the highest efficiency of putative transformant production (83 %) in treatment with no preculture, bacterial OD_{600} of 0.6 and the addition of 150 mg/l cefotaxime in the pre-selection and selection media. The presence of the *At*HSP101 in the plant genome was verified by luciferase reporter gene assay. Nested PCR amplification of genomic DNA isolated from each putative transformed plantlet showed the expected 520 and 689 bp products of *At*HSP101 and CaMV35S promoter primers, respectively. Southern blot analysis of the transgenic strawberry showed the presence of one, two and three copies of the transgene in the plant genome. Analysis of the *At*HSP101 expression at the mRNA level by RT-PCR showed the expected band of 520 bp corresponding to the *At*HSP101 cDNA. Protein dot blot and western blot analysis of the transgenic lines with one copy number of the transgene indicated the positive interaction of protein with the antibodies confirming the expression of *At*HSP101 at the protein level. DNA dot-blot analysis of the transgenic strawberry lines derived from runners and having one copy number of the transgene showed the presence of a hybridization sequence homologous to the *At*HSP101. Greenhouse evaluation of the transgenic strawberry lines exhibited robust growth and performance compared to the non-transgenic control plant. Study on the effect of two temperatures, 20 and 30 °C showed greater growth and productivity in the transgenic lines compared to the control plants. Analysis of transgenic strawberry plants exposed to heat shock, gradual heat and drought stresses showed significant differences in their morphological characters and *At*HSP101 protein level, measured by ELISA, compared to the control plants. The results of this study indicated that the expression of the *At*HSP101 may have an application to protect the transgenic strawberry plant under high temperature and drought conditions.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah doktor falsafah

PEMBANGUNAN STRAWBERI (*FRAGARIA X ANANASSA* DUCH., cv. CAMAROSA) TUMBUHAN YANG TOLERAN PEMBAWAAN HABA MELALUI TRANSFORMASI BERPERANTARAAN *AGROBACTERIUM*

Oleh

FATEMEH HADDADI

Disember 2012

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Di Malaysia pengeluaran strawberi adalah terhad di kawasan tanah tinggi. Oleh itu, berdasarkan kawasan pengeluaran yang terhad aplikasi kejuruteraan genetik untuk penghasilan strawberi toleran haba akan memudahkan penanaman tanaman tersebut di kawasan tanah rendah. Satu kaedah transformasi berperantaraan *Agrobacterium* telah diguna untuk memasukkan gen *At*HSP101 ke dalam strawberi cv. Camarosa. cDNA *At*HSP101 di bawah kawalan promoter CaMV35S diklonkan ke tapak pengklonan pelbagai pGreen0049. Vektor yang dibentuk dinamakan sebagai pGFHSP dan berjaya dipindahkan ke dalam *Agrobacterium* LBA4404. Sistem regenerasi *in vitro* daripada daun strawberi untuk digunakan dalam transformasi telah dioptimumkan melalui aplikasi pelbagai kepekatan berbeza TDZ dalam medium MS. TDZ pada kepekatan 16 µM menunjukkan peratusan tertinggi pembentukan pucuk (100 %) dan purata bilangan pucuk tertinggi (24) dihasilkan per eksplan. Kesan antibiotik berbeza iaitu timentin, sifotaksim, karbenisilin dan ampisilin terhadap regenerasi eksplan pucuk daun strawberi menunjukkan regenerasi pucuk terbaik dengan kehadiran 300 mg/l timentin dan 150 mg/l sifotaksim.

Penentuan kepekatan perencatan minimum (*MIC*) kanamisin telah dijalankan menggunakan eksplan daun untuk saringan berkesan transforman strawberi putatif. Kanamisin pada kepekatan 50 mg/l dengan peratusan regenerasi pucuk 0 % dan min bilangan 0 pucuk per eksplan telah dipilih sebagai MIC. Penilaian faktor berbeza yang mempengaruhi transformasi strawberi berperantaraan *Agrobakterium* dengan *At*HSP101 menunjukkan kecekapan tertinggi penghasilan transforman putatif (83 %) pada rawatan tanpa pra-kultur, OD_{600} bakteria 0.6 dan penambahan 150 mg/l sifotaksim pada medium pra-pemilihan dan pemilihan. Kehadiran *At*HSP101 dalam genom tumbuhan telah disahkan melalui asai gen pelapor lusiferase. Amplifikasi DNA genom yang dipencil dari daun muda setiap anak pokok transforman putatif melalui teknik '*Nested PCR'* menunjukkan produk 520 dan 689 bp masing-masing yang dijangka bagi primer *At*HSP101 dan promoter CaMV35S. Analisis *Southern blot* bagi strawberi transgenik menunjukkan kehadiran satu, dua dan tiga salinan transgen dalam genom tumbuhan. Analisis pengekspresan *At*HSP101 pada tahap mRNA yang dijalankan mengunakan teknik *RT-PCR* menunjukkan jalur 520 bp yang dijangka bersamaan dengan gen *At*HSP101. Analisis *Protein dot blot* dan *Western blot* bagi baris transgenik dengan satu salinan transgen menunjukkan interaksi positif di antara protein dengan antibodi yang sekaligus mengesahkan pengekspresan gen *At*HSP101pada tahap protein. Analisis *DNA dot blot* bagi baris strawberi transgenik dengan satu salinan transgen, yang berasal dari rayapan, menunjukkan kehadiran jujukan hibridisasi homologus *At*HSP101. Penilaian di dalam rumah hijau bagi baris strawberi transgenik mempamerkan prestasi dan pertumbuhan yang memberangsangkan berbanding dengan tumbuhan bukan transgenik sebagai kawalan. Kajian kesan dua suhu, 20 and 30 °C menunjukkan pertumbuhan dan produktiviti lebih tinggi bagi baris transgenik berbanding tumbuhan kawalan. Analisis tumbuhan transgenik strawberi yang didedahkan kepada kejutan haba, haba secara beransur-ansur dan tekanan kemarau menunjukkan perbezaan yang signifikan bagi ciri morfologi dan tahap protein *At*HSP101 berbanding dengan tumbuhan kawalan. Keputusan kajian ini menunjukkan bahawa pengekspresan *At*HSP101 erkemungkinan mempunyai aplikasi untuk melindungi tumbuhan strawberi transgenik di bawah suhu yang tinggi dan keadaan kemarau.

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APPROVAL

I certify that a Thesis Examination Committee has met on 26 December 2012 to conduct the final examination of Fatemeh Haddadi on her thesis entitled "DEVELOPMENT OF HEAT TOLERANT STRAWBERRY (*FRAGARIA X ANANASSA* DUCH., cv. CAMAROSA) PLANTS THROUGH *AGROBACTERIUM*-MEDIATED TRANSFORMATION" 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 degree of Doctor of Philosophy.

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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.

FATEMEH HADDADI

Date: 26 December 2012

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LIST OF ABBREVIATIONS

A. tumefaciens	*Agrobacterium tumefaciens*
ANOVA	analysis of variance
BA	N6-benzyladenine
BAP	6-benzylaminopurine
bp	base pair
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CRD	randomized complete design
DMRT	duncan multiple range test
E.coli	*Escherichia coli*
et al.	et alia
ELISA	enzyme-linked immune sorbent assay
GH	gradual heat
GFP	green fluorescent protein
GUS	β-glucuronidase
HS	heat shock
HSP	heat shock protein
IAA	indole-3-acetic acid
IBA	indole-3-buteric acid
OD	optical density
PCR	polymerase chain reaction
PBS	phosphate buffered saline

RLU	relative luminescence unit
RT-PCR	reverse transcription polymerase chain reaction
PGR	plant growth regulator
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sp.	species
TAE	tris-acetate/EDTA electrophoresis buffer
TDZ	thidiazuron
Tm	temperature
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
2iP	6-(α,α-dimethylallylamino)-purine
2,4-D	2,4-dichlorophenoxyacetic acid

CHAPTER 1

INTRODUCTION

There is a worldwide growing demand for strawberry due to its richness in vitamins, polyphenolics and phytochemicals, pleasant aroma and flavor, and fresh appearance. Strawberry constitutes an important part of greenhouse production, and many studies on strawberry have been conducted. Complicated genetic background contributed by high heterozygosity and polyploidy of strawberry impedes improvement of this crop through traditional breeding methods. Manipulation at gene level paved the way for further amelioration of strawberry improvement although the octoploid genome of strawberry poses difficulties for genetic and molecular studies. Studies on gene function in strawberry are few (Folta *et al.,* 2006). Furthermore concomitant application of genetic manipulation and classical breeding methods could accelerate cultivar developmental programs.

Plants are generally exposed to various stress factors and environmental conditions which affect their productivity and growth. Within the context of environmental changes, global warming or climate change as one of the abiotic stress factors has the foremost global impact on yield of crops. The optimal growth temperature for temperate species varies from 11.5 to 26 °C, whereas for tropical/subtropical species is between 23 and 32 °C (Lee *et al.,* 2007). Increase in temperature, 5–10 °C above ambient, causes damage of proteins and enzymes involved in metabolism (Porter, 2005). These changes may take a few minutes to a few hours as a result of the heat shock (Cho *et al.,* 2012; Yan *et al.,* 2011). Biological stress like elevated temperatures result in expression of heat shock proteins (HSPs) that influence survival of plants under stress conditions (Park *et al.,* 2012). HSPs are highly conserved and commonly expressed constitutively by facilitating the synthesis and folding of proteins. HSP101, a member of the HSP families, plays a critical role in acquiring thermotolerance.

Strawberry as a temperate crop is grown in the highland regions of Malaysia such as in Cameron Highlands at an altitude of 6,001ft (1,829 m) above sea-level. Cameron Highlands have the optimum weather and condition for strawberry cultivation with temperatures ranging between 13.7 °C and 23.6 °C and a mean value of 17.4 °C. Temperatures higher than 30 °C affect growth, runner production, flowering and fruiting of strawberries resulting in production of lower quality strawberries (Li *et al.,* 2010). Increment of temperature even by 1 °C was found to decrease strawberry firmness (Pyrotis *et al.,* 2012). Besides, limited availability of areas for cultivation other problems encountered in strawberry production in Cameron highlands are the sloppy lands, land degradation and distance.

Pressed by these concerns, an attempt was undertaken in this study to produce strawberry plants tolerant to high temperature stress via genetic engineering using *Arabidopsis thaliana* HSP101 (*At*HSP101) cDNA which may enable the crop to be grown at the lowland. By far the most exploited method of strawberry gene transformation is *Agrobacterium*-mediated transformation (Qin *et al.,* 2011; Mezzetti, 2009). Indeed strawberry cv. Camarosa is the most common cultivar grown in Malaysia. Hence optimization and development of *Agrobacterium*-mediated transformation of strawberry cv. Camarosa using the *At*HSP101 for heat tolerance were conducted in this study.

Therefore the main aims of this study were:

1- To determine the effect of different concentrations of TDZ and antibiotics on *in vitro* shoot regeneration from strawberry leaves

2- To construct pGreen0049 binary vector harbouring *At*HSP101 cDNA, to optimize factors affecting *Agrobacterium*-mediated transformation of strawberry with the cDNA, and to verify the presence and expression of the gene in the plant genome

3- To evaluate the performance of the transgenic plants in the transgenic greenhouse and to study the effect of abiotic stresses on the expression of the *At*HSP101 in the transgenic strawberry lines

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