Faculty of Resource Science and Technology

TRANSFORMATION OF *ISOCHORISMATE SYNTHASE* IN TOMATO PLANTS

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TRANSFORMATION OF *ISOCHORISMATE SYNTHASE* IN TOMATO PLANTS

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This project is submitted in partial fulfilment of the requirement for the Degree of Bachelor of Science with Honour in Resource Biotechnology

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DECLARATION

I declare that this thesis entitled Transformation of *Isochorismate synthase* in Tomato Plants is the result of my own research except as cited in the references. No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institutions of higher learning.

Signature: _____

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Date:

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LIST OF ABBREVIATIONS

%	percent
µl	microlitre
°C	degree Celcius
bp	base pair
AGE	Agarose Gel Electrophoresis
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
ICS	*Isochorismate synthase*
LA	Luria Agar
LB	Luria Broth
min	minute
ml	mililitre
mM	micromolar
MT1	MARDI Tomato 1
nm	nanometre
OD	Optical density
pGSA1131_*ICS*	plasmid GSA1131 harbouring *ICS*
PCR	Polymerase Chain Reaction
RE	restriction enzyme
Ri-plasmid	Root inducing-plasmid
rpm	rotation per minute
sec	second
T-DNA	transfer DNA
Ti-plasmid	Tumor inducing-plasmid
vir	virulence gene

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Tranformation of *Isochorismate synthase* into Tomato Plants

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ABSTRACT

Isochorismate synthase (ICS) is an important enzyme during the conversion of chorismate into isochorismate in the shikimate pathway. The cDNA was transformed into tomato plant to observe the functional and expression activity. The objective of this study is to insert the *ICS* gene isolated from *Morinda citrifolia* into tomato by seed via infiltration method. Mardi Tomato 1 (MT1) seeds were treated with sonication before immersed in infiltration broth on a shaking incubator and subsequently sown into soils. About 887 bp size of *ICS* cDNA was not successfully transformed into the MT1 plants confirmed by polymerase chain reaction (PCR).

Key words: *ICS*, infiltration, tomato, *Agrobacterium tumefaciens*, seed

ABSTRAK

Isochorismate synthase (ICS) merupakan enzim yang penting untuk penukaran chorismate kepada isochorismate dalam laluan shikimate. cDNAnya telah ditransform ke dalam pokok tomato untuk melihat aktiviti kefungsian dan ekspresinya. Objektif kajian ini adalah untuk memasukkan gen ICS yang telah diasingkan daripada Morinda citrifolia ke dalam biji benih tomato melalui kaedah infiltrasi. Biji benih Mardi Tomato 1(MT1) dirawat dengan ultrabunyi sebelum direndam ke dalam medium infiltrasi di atas incubator bergerak dan kemudiannya disemai ke tanah. cDNA ICS yang bersaiz kira-kira 887 bp tidak berjaya ditransform ke dalam pokok tomato berdasarkan reaksi berantai polymerase.

Kata kunci: ICS, infiltrasi, tomato, Agrobacterium tumefaciens, biji benih

1. INTRODUCTION

Plant transformation is defined as the insertion and incorporation of foreign deoxyribonucleic acid (DNA) into the plant cells in order to produce plants with additional new genes (Newell, 2000). Nowadays, there are a lot of techniques to introduce foreign genes into a host plant cells such as the *Agrobacterium*-mediated transformation, particle bombardment, electroporation, silicon carbide fibres, microinjection and virus-based transformation (Darbani *et al*., 2008).

There are two universally used strains of Agrobacteria involve in plant transformation, which are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. They carry Ti (tumor-inducing) plasmid and Ri (root-inducing) plasmid respectively. As cited in Darbani *et al*. (2008), *A. rhizogenes* is more suitable for studies on recalcitrant species of plants (Tepfer, 1984), production of artificial seeds (Uozomi & Kobayashi, 1997) and secondary metabolites (Hamill & Lidgett, 1997). In this research, *Agrobacterium tumefaciens* was utilised to transform *isochorismate synthase* (*ICS*) cDNA into tomato seeds via infiltration method.

ICS is an enzyme that is commonly associated with the accumulation of salicylic acid in plants due to the plant response against pathogens. Chorismate, a substance in the shikimate pathway will be converted into isochorismate through the catalysis of ICS (van Tegelen *et al.*, 1999). This process will eventually produce salicylic acid and other products as well such as 2,3-dihydroxybenzoate and *ortho*-succinylbenzoate (Strawn *et al*., 2007). As *ICS* encoding cDNA was previously isolated from *Morinda citrifolia*, it is important to know if this cDNA is able to express and function in other type of plants.

We used tomato as the host plants the transformation in this research. It is chosen as the host plant because the seeds are easily available. Besides, a lot of research studies

have been done on tomatoes and the information are greatly accessible which makes it a good host plant in this research. Several parts of tomato have been used for transformation studies such as leaf disc (McCormick *et al*., 1986), seed (Sharma *et al.,* 2009) and fruit (Hasan *et al*., 2008).

The objective of this research project is to transform the cDNA of *isochorismate synthase,* which the gene was isolated from *Morinda citrifolia*, into tomato plants.

2. LITERATURE REVIEW

2.1 *Agrobacterium*-mediated transformation

Agrobacterium is a gram-negative bacterium and belongs to the category of Eubacteria. The first attempt to transfer the bacterial DNA into the plant cell using *Agrobacterium* had been initiated in 1977 by Chilton *et al*. (1977). The infection of the soil bacteria into the host plant cell causes a disease called crown gall disease which can be seen at just above the soil level. Until today, transformation via *Agrobacterium tumefaciens* has been the most widely utilized compared to other techniques of plant transformation such as silicon carbide whiskers, electroporation, particle bombardment and desiccation based transformation. The infestation of *A. tumefaciens* into the host plants will also cause the production and secretion of opines, the sugar and amino acid derivatives which can never be found in healthy plants (Tourte, 2005). Opines serve as the nutrient to the bacteria and the bacteria will feed on them. The advantages of transforming foreign gene into plants by *Agrobacterium* are stable gene expression, low cost, high transformation efficiency and larger size of DNA can be inserted.

The examples of crops that have been successfully transformed by *Agrobacterium tumefaciens* are wheat (Chugh *et al.*, 2012), *Arabidopsis thaliana* (Valvekens e*t al.*, 1988), tomato (Sharma *et al*., 2009) and *Musa* spp. (Tripathi *et al.*, 2005). Genetic modification in plants has cause a lot of benefits including delay in fruit ripening, resistance towards antibiotics, herbicides and diseases, production of foreign proteins and characterisation of protein function.

2.1.1 T-DNA of Ti-plasmid

A. tumefaciens infection in the wound site of plants generally would cause the formation of neoplastic growth, known as the crown gall disease caused by Ti plasmid. The Ti plasmids

in *A. tumefaciens* usually 200-800 bp in size consists of several parts which are *vir* region, T-DNA, opine breakdown region and origin of replication. The only region that can be transferred into the plant cell is the T-DNA. It is flanked by 25 bp sequences located at left and right side of the insert which are called left border and right border, respectively (Draper & Scott, 1991). The right border is more crucial than the other one for an efficient T-DNA transfer.

T-DNA comprises left and right border, genes encoding for opine, auxin and cytokinin synthesis. Auxin and cytokinin cause the proliferation of the tumor cells at the wounded area whereas opines, the amino acid and sugar phosphate derivatives become the carbon and nitrogen sources for the bacterium to grow (Anand & Mysore, 2007).

2.1.2 Binary vector

Binary-vector, which is also known as trans-acting *vir* genes system involves two types of plasmids, in which the plasmid harbouring T-DNA (binary vector) and plasmid containing *vir* genes (*vi*r helper) are separated. When both plasmids are placed in one *Agrobacterium* cell, the *vir* gene products can act on the T-region so that the gene of interest can be excised and inserted into the plant host genome. The *vir* helper plasmid usually has complete or partial deletion of T-DNA region, thus circumventing the tumorigenesis which the scientists desired as they wanted to produce a normal plant in their research (Lee & Gelvin, 2008). One limitation for a transgenic plant with tumor-inducing gene is it cannot produce normal shoots (Draper & Scott, 1991). Thus, scientists generate the non-oncogenic plasmids by replacing the gene with selectable markers such as antibiotic for selection of transformants. The examples of *Agrobacterium* strains that have been modified with *vir* helper plasmids are LBA4404, GV3101 MP90 and AGL0 (Gelvin, 2003).

2.1.3 Mechanism of T-DNA entry into the plant host cell

There are six *vir* genes present in the vir region, which are *vir* A, B, C, D, E and G. The transfer of T-DNA from Ti-plasmid to the plant host nuclear genome is majorly mediated by *vir* genes activity (Zupan & Zambryski, 1995). Loci A, B, D and G are important for transfer of the T-DNA whereas C and E are essential for the transfer efficiency. The left and right borders of T-DNA become the target for VirD1/VirD2 border-specific endonuclease to excise the fragment from the *Agrobacterium* (Gelvin, 2003).

During the infection process of *A. tumefaciens* to the host plant, the bacterium will attach to the wounded area thus *vir*A gene is activated. The activation of the gene is triggered by the plant phenolic compound at the wound site such as acetosyringone where the VirA protein can sense (Gelvin, 2003). This activates *vir*G gene as well and subsequently VirG protein facilitates the transcription of *vir* genes in Ti-plasmid. The product of the transcription renders the T-DNA excision and forms a T-complex. A channel will be created to allow the entry of T-complex into the plant host cell.

2.2 Isochorismate synthase

Isochorismate synthase (ICS) is an enzyme that catalyses the reversible conversion of chorismate into isochorismate in shikimate pathway (Strawn *et al.*, 2007). Chorismate can be found in bacteria, fungi and plants to produce primary metabolites such as amino acids and other products. In *Bacillus subtilis*, ICS is involved in the synthesis of respiratory chain component menaquinone and siderophore 2,3-hydroxybenzoate (Rowland & Taber, 1996). Unlike plants, the information of isochorismate in bacteria is more advanced than in plants as the enzymes, products derived from them and their functions are still lacking. It was previously predicted that ICS is located at the plastid of the plant due to the presence

of chloroplast plastid sequence predicted and the fact that chorismate is synthesized in the plastid as confirmed by a study conducted by Strawn *et al.* (2007).

In chorismate metabolism, one of the important product compounds that can be obtained in plant is salicylic acid. Salicylic acid is a plant hormone and vital in plant defence system (Wildermuth e*t al.*, 2001). Besides that, plant with salicylic acid has been reported to be used for medicinal purposes. During 5th century, willow leaf and bark which is rich in salicylate had been used as pain reliever during childbirth (Rainsford, as cited in An & Mou, 2011). Salicylic acid has been reported to intensify the stomatal closure in *Commelina communis* (Rai *et al.,* 1986), *Vicia faba* (Manthe *et al*., 1992) and induce the flowering of *Lemna gibba* G3 (Tanaka & Cleland, 1980). There has been no attempt to insert *ICS* encoding cDNA into tomato so far.

2.3 Tomato (*Solanum lycopersicum*)

Tomato belongs to the Solanaceae family, along with potato, tobacco and eggplants. It is one of the major vegetable crops in the world and commonly used as a genetic model in plants enhancement (Arumuganathan & Earle as cited in Sharma *et al*., 2009). The tomato fruits can be round, pear-like shaped, plum-like shaped, oval or long. They are classified under berries due to the many little seeds covered in the thick pericarps (Grandillo *et al.*, 1999) and categorised under climacteric type of fruits which the ripening is controlled by the production of ethylene and carbon dioxide (Seymour *et al.*, 2012).

In 1986, McCormick *et al.* made the first attempt to utilise tomato in transformation research via leaf disc. There are various studies done on tomato which makes it an important plant material for research includes improved transformation protocol (Chaudhry & Rashid, 2010), genetic control on the development of fruit (Grandillo *et al.*, 1999),

genetic control of the tomato features (Paran & van der Knaap, 2007) and ripening control (Seymour *et al.*, 2012). One of the most famous contributions of tomato in genetic engineering of plant is the „Flavr Savr' tomato which it is proven to be more solid and much firmer compared to the other varieties that lead to a longer shelf life (Halford, 2006).

3. MATERIALS AND METHOD

3.1 Preparation of seeds

3.1.1 Treatment of seeds

About 100 seeds of MARDI Tomato 1 (MT1) tomato were washed and put into a Falcon tube. Distilled water was added into the tube until the seeds were fully immersed. The tube was placed in a fridge of 4°C overnight.

3.1.2 Infiltration

After imbibitions of seeds, the seeds were transferred into a conical flask and placed in a sonicator in Physical Chemistry Lab for 8 minutes at room temperature. Then, the distilled water was discarded and replaced with 60 ml infiltration broth added with acetosyringone and silwet. The flask was placed on an 80 rpm shaker for 3 hours at room temperature.

3.1.3 Post-Infiltration

After three hours shaking, about 100 μg/ml of carbenicillin was added into the infiltration broth. Then, the infiltration broth was drained from the tomato seeds and the seeds were rinsed with distilled water for 2 times. Then, about 6 to 7 seeds were sown into 15 polybags of soil and exposed to normal daylight. The seeds were watered once a day.

3.2 Preparation of *A. tumefaciens* harbouring pGSA1131

3.2.1 Competent *E. coli* XL1-Blue cells preparation

The competent cells of *E. coli* XL1-Blue was prepared using calcium-chloride ($CaCl_2$) method. About 200 μl of *E. coli* XL1-Blue from stock culture was inoculated into a 15 ml Falcon tube containing 5 ml of LB medium. It was cultured an overnight with 250 rpm shaking at 37°C.

On the next day, 100 µl of the overnight culture was transferred into 10 ml LB. It was allowed to grow at 37°C with shaking at 250 rpm for about 1 hour and 20 minutes. Using a spectrophotometer, the OD_{600} of the culture was determined until the value of 0.45 to 0.5 was reached.

The Falcon tube was placed on ice for 10 to 20 minutes. The cell suspension was centrifuged at 3500 rpm for 5 minutes at 4°C. The supernatant was discarded and the cells were washed by resuspending them in 15 ml iced-cold 100 mM calcium chloride ($CaCl_2$). It was done by pipetting up and down gently and kept on ice for 10 minutes. After that, the suspension was re-centrifuged at 3500 rpm for 3 minutes at 4°C. The supernatant was discarded again and resuspended in 1 ml of cold sterile 100 mM $CaCl_2$. About 200 µl of the glycerol and 800 µl of the mixture from the Falcon tube were mixed and put into 50 µl aliquots in 0.5 ml microcentrifuge tubes. Then, the cells were kept in -80°C freezer until use.

3.2.2 Isolation of pGSA1131

About 500 µl of *E. coli* XL-1 Blue containing pGSA1131 with *ICS* (pGSA1131_*ICS*) and pGSA1131 without *ICS* from the stock cultures were cultured into 10 ml LB medium which also added with 30 µg/ml chloramphenicol antibiotic into two different tubes. They were cultured overnight while shaking. Then, the plasmids were isolated using Gene MATRIX Plasmid Miniprep DNA Purification Kit and checked using AGE analysis. It was visualised under UV transilluminator and the photograph was taken using GelDoc.

3.2.3 Restriction enzyme digestion of binary constructs

The binary constructs with and without *ICS* were digested using NcoI restriction enzyme (RE) to compare the sizes of the linearised plamids. In two separated 0.5 ml microcentrifuge tubes, 8 µl plasmids were mixed with 1 µl *Nco*I and 1 µl Tango buffer.

The mixtures were incubated at 37°C overnight. On the next day, the reaction was stopped at 65°C using a heat block for 10 minutes. The digested plasmid were analysed by 1% AGE and visualised under UV transilluminator. The gel photo was taken using GelDoc.

3.2.4 Polymerase Chain Reaction

The linearised binary constructs were further checked with polymerase chain reaction (PCR) which confirmed that the plasmid contain *ICS* cDNA. PCR was carried out using Bio-Rad iCycler to detect the presence of *ICS* gene following the composition mixture and parameter as in Table 3.1 and Table 3.2 below. The primers used were ICSha1F (5' GCA TTG GCC ATG GAA CGT CT 3') and ICSha3R (5' TCT GGA GTG TTT CCA ATG AAT GC 3'). The PCR products were analysed with 1% agarose gel and the photo was taken using GelDoc.

Table 3.1: Amplification composition of PCR mixture

Ingredients	Volumes (µl)
GoTaq	7.5
ICSha1F	1
ICSha3R	1
Template	1
Nuclease Free Water	4.5
Total	15

Table 3.2: PCR amplification parameter

	Steps	Temperature (°C)	Duration
Step 1	Initial denaturation	94	3 min
Step 2	Denaturation	94	30 sec
Step 3	Annealing	59	30 sec
Step 4	Extension	72	45 sec
Step 5	Repeat Step 2-4 for total 35 cycles	72	2 min
Step 6	Holding	20	∞

3.2.5 Transformation of pGSA1131 into competent *E. coli*

The competent cells were thawed on ice for 10 minutes with the plasmid mixture. About 10 µl of plasmid DNA was added into 50 µl of the competent cells in a 0.5 ml microcentrifuge tube and incubated on ice for another 10 minutes.

The tube was put into 42°C water bath for 2 minutes for heat shock and into ice for another 10 minutes. The mixture was transferred into pre-warmed 1 ml of LB in 37°C water bath and incubated for 60 minutes at 37°C on a shaker.

About 250 µl of the *E. coli* culture was plated out on LA medium with 30 µg/ml of chloramphenicol for selection. The plates were incubated overnight at 37°C incubator.

3.2.6 Colony PCR

Each single colony grown on the LA medium was re-cultured into 1 ml LB medium in a 1.5 ml microcentrifuge tube with the same concentration of chloramphenicol as above. It was cultured for an overnight on a 150 rpm shaker.

About 700 µl of the overnight culture was inoculated into another 1.5 ml microcentrifuge tube to be analysed in colony PCR whereas the remaining was kept for stock. It was centrifuged for 13500 rpm within 6 minutes at room temperature. The supernatant was then discarded and the pellet was resuspended with 500 µl sterile water.

The tube was heated at 89 to 90°C for 8 minutes. It was vortexed three times within the period of time and re-centrifuged with the same condition as above. The supernatant was transferred into a new tube can be used in PCR reaction. PCR was done using the same composition of PCR mixture and parameter as in Table 3.1 and Table 3.2.

3.2.7 Preparation of electrocompetent *A. tumefaciens* LBA4404

The preparation of electrocompetent *A. tumefaciens* protocol was adapted from Bio-Rad Gene Pulser Xcell Electroporation System Instruction Manual. About 10 µl of *A.*

tumefaciens was cultured within 48 hours in 20 ml LB medium and 100 µg/ml of rifampicin concentration. It was incubated at room temperature on a 180 rpm shaker. Then, 1 ml of the overnight culture was inoculated into 10 ml LB and incubated again until OD_{550} of about 1.0 was reached.

Next, the culture was centrifuged at 3000 rpm and 4°C within 10 minutes. The supernatant was carefully discarded and the Falcon tube containing the pellet was put on ice. About 1 ml of sterile, ice-cold 10% glycerol was added into the tube. The cell pellet was vortexed to resuspend it. The steps of centrifugation and addition of glycerol were repeated for another one time.

Then, the pellet was resuspended in 1 ml of sterile, ice-cold 10% glycerol. About 50 µl of the cell was aliquot into 0.5 ml microcentrifuge tubes and stored in -80°C until use.

3.2.8 Transformation of pGSA1131_*ICS* into *A. tumefaciens* LBA4404

The transformation of pGSA1131 into *A. tumefaciens* was done by electroporation using an adapted protocol from Bio-Rad Gene Pulser Xcell Electroporation System Instruction Manual.

The frozen competent cell of 50 µl *A. tumefaciens* and pGSA1131_*ICS* were thawed on ice. About 1.5 µl the DNA plasmid was added into the 50 µl of *A. tumefaciens* competent cell for mixing. All contents in the tube was taken out using a micropipette with small tip and placed into a small hole in the pre-cooled Bio-Rad Gene Pulser Cuvette.

The *Agrobacterium* competent cell in the brown cuvette was electroporated using the Bio-Rad GenePulser Xcell machine. After that, 1 ml LB was added into the cuvette immediately and the content was mixed gently by pipetting up and down. The mixture was

transferred into 1.5 ml microcentrifuge tube and incubated at room temperature with vigorous shaking about 3 hours until the culture turned cloudy.

Then, about 250 µl of the *Agrobacterium* culture was spread on the LA medium containing 50 µg/ml rifampicin and 30 µg/ml chloramphenicol. The plate was placed upside down and covered with an aluminium foil. It was incubated on the workbench for two days in room temperature.

3.2.9 The isolation of pGSA1131 harbouring *ICS* from *A. tumefaciens*

A single colony grown on the LA medium was cultured in 5 ml LB medium for an overnight which containing the same concentration of rifampicin and chloramphenicol as the above protocol. The plasmid GSA1131 harbouring *ICS* was isolated using Gene MATRIX Plasmid Miniprep DNA Purification Kit. The plasmid isolated was subjected to agarose gel electrophoresis (1%) and it was viewed under UV transilluminator. The photo of the bands produced was taken using GelDoc. Consequently, PCR was carried out using the same profile as in 3.2.4 to detect the presence of *ICS*.

3.3 Preparation of *Agrobacterium* for seeds infiltration

About 500 µl the verified colony culture was re-cultured into 15 ml LB medium and incubated at 37°C, with 180 rpm shaking for two days. Approximately, 4 ml of the overnight culture was added into 30 ml of LB medium containing 50 µg/ml rifampicin and 30 µg/ml chloramphenicol. It was cultured on a 180 rpm shaker until OD_{600} value of about 0.5 to 0.6 was reached, around two and a half hours. Then, the culture was centrifuged at 3500 rpm for 8 minutes at room temperature. The cells were resuspended in 60 ml infiltration media.

3.4 Analysis of putative transformants

3.4.1 Extraction of plant genomic DNA

The extraction of plant genomic DNA was done by using the protocol by Edwards *et al.* (1991). About 0.1-0.5 mg of the tomato plants leaves were cut using sterilized a blade and placed into 1.5 ml microcentrifuge tubes. Then, the tubes were snapped frozen in liquid nitrogen and ground into fine pieces by pestle stick. About 450 µl of extraction buffer was