OPTIMISATION OF TRANSFORMATION SYSTEM AND EXPRESSION OF A CINNAMATE-4-HYDROXYLASE (C4H) GENE SILENCING CONSTRUCT IN SUSPENSION CELLS OF *BOESENBERGIA ROTUNDA*

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Field of Study: Plant Molecular Biotechnology

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ABSTRACT

Boesenbergia rotunda (L.) Mansf. also known as the fingerroot ginger or "Temu kunci" in Malay, produces valuable pharmaceutical compounds including panduratin A, 4'-hydroxypanduratin A, pinostrobin, pinocembrin chalcone, pinocembrin, isopanduratin A and cardamonin. In this study, an enzyme involved in the pathway responsible for biosynthesis of these compounds, cinnamate-4-hydroxylase (C4H) was partially cloned and a double-stranded RNA (dsRNA) construct was introduced for knockdown/ RNAi of the enzyme expression in *B. rotunda* cell suspension culture. Prior to the RNAi of the enzyme, a *B. rotunda* cell suspension culture and *Agrobacterium*-mediated transformation system was developed and optimised. The highest specific growth rate of the cell suspension was recorded as 0.0892±0.0035 in Murashige and Skoog liquid media supplemented with 1.0 mg L^{-1} of 2,4-dichlorophenoxyacetic acid and 0.5 mg L^{-1} 6-benzyladenine, representing a 12-fold increase in cell volume during the culture period. Parameters affecting *Agrobacterium*-mediated transformation of the cell i.e. selection agent (hygromycin B) doses, co-cultivation periods and infection times were assessed. Optimal transformation efficiency was achieved when *B. rotunda* suspension cells were infected with *Agrobacterium tumefaciens* harbouring pCAMBIA1304 for 10 min and co-cultivated for 2 days. Polymerase Chain Reaction (PCR) and Southern hybridization analysis revealed stable integration of *mgfp5* gene in the cell suspension culture up to 12-mo of maintenance and subculture. Out of 66 cell lines transformed with *Agrobacterium* carrying the C4H-dsRNA RNAi vector screened via PCR analysis, one cell line was obtained and Southern analysis confirmed the presence of *gusI* gene that functions as a hairpin loop in the RNAi vector. Quantitative-Reverse transcription PCR (qRT-PCR) analysis revealed

the expression level of C4H transcripts in the RNAi cell line was 2-fold lower than wild type cells. The presence of homologous small RNAs in northern blot analysis but absence in the wild type confirmed that the knockdown was triggered by the dsRNA introduced. Differential expression of primary and secondary metabolites profiles were revealed via Liquid Chromatography Mass Spectrum (LC-MS) analysis. In conclusion, RNAi of the enzyme C4H via a partial hairpin dsRNA has provided insights into the functions and channels in the biosynthesis pathway involving the enzyme C4H which shown in this study, is non-redundant in biosynthesis of secondary metabolites in *B. rotunda* cell suspension. *B. rotunda* cell suspension could serve as a good system for secondary metabolite pathway study as well as compound production.

ABSTRAK

Boesenbergia rotunda (L.) Mansf. dikenali sebagai "Temu kunci", menghasilkan sebatian-sebatian farmaseutikal yang berharga, termasuk panduratin A, 4'-hydroxypanduratin A, pinostrobin, pinocembrin chalcone, pinocembrin, isopanduratin A dan cardamonin. Dalam kajian ini, gen separa bagi satu enzim yang terlibat dalam laluan biosintesis sebatian-sebatian ini, cinnamate-4-hydroxylase (C4H) telah diklonkan dan digunakan untuk merendahkan ekspresi enzim ini dalam kultur pengampaian sel *B. rotunda*. Sebelum itu, kultur ampaian sel dan sistem transformasi *Agrobacterium* bagi *B. rotunda* telah dioptimakan. Kadar pertumbuhan sel ampaian yang paling tinggi direkodkan adalah 0.0892 ± 0.0035 dengan menggunakan Murashige dan Skoog media cecair yang ditambah dengan 1.0 mgL^{-1} 2,4-diklorofinosiasetik dan 0.5 mgL^{-1} 6-benziladenin, iaitu 12 kali ganda bertambah bagi jangka masa pertumbuhan sel. Parameter transformasi *Agrobacterium* iaitu dos ejen pemilihan (hygromycin B), tempoh ko-kultur dan jangkitan telah dinilai. Kecekapan transformasi yang optima dicapai apabila sel ampaian *B. rotunda* dijangkiti dengan *Agrobacterium* yang mengandungi pCAMBIA1304 selama 10 min dan diko-kultur selama 2 hari. Reaksi rantai polimerasi (PCR) dan analisis penghibridan Southern telah menunjukkan integrasi stabil gen *mgfp5* dalam kultur ampaian sel selama 12 bulan. Satu daripada 66 titisan kultur ampaian yang telah diuji melalui analisis PCR, didapati mengandungi vektor RNAi C4H-dsRNA. Penghibridan Southern yang dijalani atas titisan kultur sel tersebut mengesahkan kehadiran gen *gusI* dalam vektor RNAi. Analisis Kuantitatif-Reverse transkripsi PCR (qRT-PCR) menunjukkan tahap ekspresi transkrip C4H dalam titisan kultur ampaian sel RNAi tersebut menunjukkan 2 kali ganda lebih rendah daripada sel-sel jenis liar, iaitu control. Kehadiran RNA kecil homolog yang dijumpai

dalam analisis northern blot mengesahkan bahawa RNAi itu dicetuskan oleh dsRNA yang digunakan dalam eksperimen. Perbezaan ekspresi dan profil metabolit primari dan sekunder telah dikaji melalui analisis Liquid Chromatography Mass Spectrum (LC-MS). Sebagai kesimpulannya, RNAi C4H enzim dengan menggunakan hairpin RNA dalam kajian ini telah menyumbangkan maklumat mengenai fungsi dan saluran di laluan biosintesis yang melibatkan C4H enzim ini. Ia adalah penting dalam biosintesis metabolit sekunder dalam ampaian sel *B. rotunda*. Selain itu, ampaian sel *B. rotunda* boleh berfungsi sebagai sistem yang berguna untuk kajian laluan metabolit sekunder dan juga sebagai penghasilan sebatian-sebatian tersebut.

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"Lord Jesus, worthy of all the praise."

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
g	:	Gram
Mg	:	Milligram
ml	:	Milliliter
µ	:	Micro
µl	:	Microliter
µmol	:	Micromole
°C	:	Degree Celcius
%	:	Percent
-ve	:	Negative
AA	:	Amino Acids
ADC	:	Arginine Decarboxylase
AFLP	:	Amplified Fragment Length Polymorphism
AGO	:	Argonaute
ANOVA	:	Analysis of Variance
ANR	:	Anthocyanin Reductase
BAP	:	6 – Benylaminopurine
BBE	:	Berberine Bridge Enzyme
BCIP-T	:	5-Bromo-4-Chloro-3-Indolyl Phosphate, p-Toluidine Salt
bp	:	Base Pair
CaMV	:	Cauliflower Mosaic Virus
CAPE	:	Caffeic Acid Phenethyl Ester
CDKs	:	Cyclin-Dependent Kinases

cDNA	:	Complementary DNA
CGA	:	Chlorogenic Acid
CHI	:	Chalcone Isomerase
CHS	:	Chalcone Synthase
chv	:	Chromosomal Virulence Genes
CIP	:	Calf Intestinal Phosphate
CFU	:	Colony Forming Unit
cm	:	Centimetre
CNTRL	:	Control
CPMP	:	Coat protein mediated protection
CTAB	:	Cetyltrimethyammonium bromide
C4H	:	Cinnamate – 4 – hydroxylase
DCL	:	Dicer-like
DFR	:	Dihydroflavonol 4-reductase
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic
DNase	:	Deoxyribonuclase
dNTP	:	Deoxynucleotriphosphate
dsRNA	:	Double-stranded RNA
EDTA	:	Ethylenediaminetetraacetic acid
EM	:	Embryogenic masses
ESI	:	Electrospray ionization
EST	:	Expressed sequence tags
EtOH	:	Ethanol
et al.	:	*Et alia*
EtBr	:	Ethidium bromide

FA	:	Formic acid
FAA	:	Formalin/ Acetic/ Alcohol
FWD	:	Forward
g	:	Gram
GABA	:	Gamma-aminobutyric acid
GFP	:	Green fluorescent protein
GPC	:	Glutaraldehyde-paraformaldehyde-caffeine
GUS	:	β-glucuronidase
HCl	:	Hydrochloride acid
hpRNA	:	Hairpin RNA
HQT	:	Hydroxycinnamoyl CoA: quinate hydroxycinnamoyl transferase
HYG	:	Hygromycin B
IAA	:	Indole-3-acetic acid
ITS	:	Internal transcribed spacer
J	:	Joule
kb	:	Kilo basepairs
kV	:	Kilo Volt
L	:	Liter
LB	:	Left borders
LC-MS	:	Liquid Chromatography-Mass Spectrometry
LD	:	Lethal dose
LM	:	Liquid media
M	:	Molar
MeOH	:	Methanol
mg	:	Miligram
$MgCl_2$	:	Magnesium chloride

MIC	:	Minimal inhibitory concentration
Mins	:	Minutes
miRNA	:	Micro ribonucleic acid
ml	:	Milliliter
mM	:	MiliMolar
mm	:	Millimetre
mRNA	:	Messenger ribonucleic acid
MS	:	Murashige and Skoog
MUG	:	4-methylumbelliferyl-β-glucuronide
MSO	:	MS media without plant growth regulator
NAA	:	α-naphthalene acetic acid
N_2	:	Nitrogen
$NaCO_3$	:	Sodium carbonate
NaOH	:	Sodium hydroxide
NaCl	:	Sodium chloride
ng	:	Nanogram
nf-H_2O	:	Nuclease-free water
NLS	:	Nuclear localization signal
NPC	:	Nuclear pore complex
nt	:	Nucleotide
OD	:	Optical density
OFN	:	Oxygen-free nitrogen blower
OrgA	:	Organic Acids
P	:	Phosphates
PA	:	Peptide amino acids
PAL	:	Phenylalanine ligase

PCR	:	Polymerase chain reaction
PEM	:	Pre-embryogenic masses
PGR	:	Plant growth regulator
pmol	:	Pico mole
PPO	:	Polyphenol oxidase
PTGS	:	Post-transcriptional gene silencing
qPCR	:	Quantitative PCR
QTL	:	Quantitative trait loci
RACE	:	Rapid Amplification of cDNA Ends
RAPD	:	Random Amplified Polymorphic DNA
RB	:	Right borders
RISC	:	RNA-induced Silencing Complex
RNA	:	Ribonucleic acid
RNAi	:	RNA interference / RNA silencing
RNase	:	Ribonuclease
REV	:	Reverse
rpm	:	Rotation per minute
R/A	:	Relative abundance
s	:	Second
SCV	:	Settled Cell Volume
SD	:	Standard Deviation
SDS	:	Sodium Dodecyl Sulphate
SE	:	Somatic embryogenesis
siRNAs	:	Small interfering RNAs
SLS	:	Secologanin synthase
SM	:	Selection media

SMA	:	Solidified selection media
spp	:	Subspecies
SRS	:	Substrate recognition sites
ss	:	Single-stranded
SSC	:	Sodium Chloride Sodium Citrate
SSCP	:	Single Strand Conformation Polymorphism
TAE	:	Tris Acetate EDTA
TAP	:	Tobacco acid pyrophosphatase
TBE	:	Tris Boric Acid EDTA
TE	:	Tris EDTA
TEMED	:	Tetramethylethylenediamine
TEV	:	Tobacco etch virus potyvirus
Tm	:	Annealing Temperature
T-DNA	:	Transferred DNA
U	:	Unit
UV	:	Ultra Violet
V	:	Volt
VIGS	:	Virus-Induced Gene Silencing
vir	:	Virulence genes
vol	:	Volume
v/v	:	Volume over volume
w/v	:	Weight over volume
X-Gluc	:	5-bromo-4-chloro-3-indolyl-β-glucuronide
YEB	:	Yeast Extract Broth
2,4-D	:	(2,4-dichlorophenoxy) Acetic Acid
4 CL	:	4 – Coumarate: Coenzyme A Ligase

4-MU	:	4-Methylumbelliferone
6- BA	:	6- Benzyladenine

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Morphology description, genetic composition and taxonomic classification of *Boesenbergia rotunda*

Boesenbergia rotunda (L.) Mansf. belongs to the *Zingiberaceae* family, originating from India and South China. The plant is commonly known as Chinese keys or fingerroot ginger in English while locally given the name "Temu Kunci" (Tan *et al.*, 2006). It is a small perennial plant (about 15 – 40 cm in height) with light green leaves and maroon-red leaf sheath. The rhizome is usually buried underground with several slender and long tuber sprouts formed out in the same direction like a bunch of keys or the fingers of a hand, thus the names "kunci" (which means keys in Malay) and fingerroot ginger. The rhizome is usually yellow in colour but some varieties have red and black rhizomes. Similar to other gingers and turmerics, the rhizome is the most widely used part of the plant.

The genome of *B. rotunda* (2n = 36) was determined by Eksomtramage *et al.* (2002). Zingiberales plant taxonomy is well-characterized with molecular marker studies such as nuclear internal transcribed spacer (ITS) (Kress *et al*., 2002), random amplified polymorphic DNA (RAPD) (Vanijajiva *et al*., 2005), amplified fragment length polymorphism (AFLP) and single strand conformation polymorphism (SSCP) (Techaprasan *et al*., 2008). These molecular methods have provided a better understanding of the phylogenetic relationships of the species in the Zingiberacea family.

1.2 Medicinal uses of *B. rotunda*

B. rotunda is commonly used as spice or food ingredient in many Asian countries due to its' aromatic flavour. It is also used as a traditional medicine to treat illnesses such as rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia and peptic ulcer, removing blood clots and as a tonic for women after childbirth (Tan *et al.*, 2012). The fresh rhizomes are used to treat inflammatory diseases, such as dental caries, dermatitis, dry cough and cold, tooth and gum diseases, swelling, wounds, diarrhoea, dysentery, and as a diuretic (Chuakul and Boonpleng, 2003; Salguero, 2003). Besides, it is also used as an antifungal and anti-parasitic agent to heal fungal infections and eradicate helminth or round worms in the human intestine, as well as an anti-scabies agent to relieve skin itchiness from mite bites (Riswan and Sangat-Roenian, 2002). In Thailand, it is referred to as "Thai ginseng" and used to alleviate food allergies and poisoning as well as an aphrodisiac, among Thai folk. In addition, it has been used as self-medication by AIDS patients (Tan *et al.*, 2012).

1.3 Pharmaceutical properties and functions

Studies have found a number of potentially valuable compounds in extracts of *B. rotunda*. Most are cyclohexenyl chalcone derivatives, flavones and flavanoids, secondary metabolites that play important roles in plant defence against UV and pathogens, pigment synthesis, fruit, flower and seed formation (Forkmann and Martens, 2001), and are also important in plant fertility and sexual reproduction (Schijlen *et al*., 2007). The active compounds include panduratin A, 4'-hydroxypanduratin A, pinostrobin, pinocembrin chalcone, pinocembrin, isopanduratin A and cardamonin which have been reported to possess anti-inflammatory (Tuchinda *et al.*, 2002), anti-mutagenic (Trakoontivakorn *et al.*, 2001), and antibacterial activities. Additionally, Tewtrakul *et al*. (2003) have found that pinostobin, pinocembrin, cardamonin and

alpinetin isolated from the ethanol extract of the closely related *Boesenbergia pandurata* Holtt. exhibited appreciable activity against HIV protease. Morikawa *et al.* (2008) isolated eight new compounds from rhizomes of *B. rotunda*: Among 18 known constituents, 4 new prenylchalcones (krachaizin A and krachaizin B) and 4 new prenylflavones (rotundaflavones), Krachaizin B, Isopanduratin, 4-hydroxypanduratin A and alpinetin showed significant inhibitory effects on TNF-α-induced cell death in L929 mouse cells (Morikawa *et al*., 2008). Moreover, Tan *et al.* (2006) demonstrated that *B. rotunda* extract has inhibitory activity against the Dengue NS2b/3 protease which is mandatory for viral replication and hence presents a potential to be developed as an anti-viral agent against this important disease. Amongst the compounds tested, panduratin A and 4-hydroxypanduratin A showed higher anti-dengue activity than the other six compounds (i.e. pinostrobin, pinocembrin, pinocembrin chalcone, caldamonin, alpinetin and isopanduratin A).

1.4 The phenylpropanoid pathway and the enzymes involved in the pathway

Chalcone derivatives are products from the phehylpropanoid pathway, which is responsible for biosynthesis of many flavanoids, flavones and chalcones. Fig. 1.1 summarises the biosynthesis of the compounds in the pathway being catalysed by several important enzymes including Phenylalanine ligase (PAL); 4 – Coumarate: coenzyme A ligase (4 CL); Chalcone Synthase (CHS); Cinnamate – 4 – Hydroxylase (C4H); and Chalcone Isomerase (CHI). The pathway starts with a simple precursor, phenylalanine which is converted into trans-cinnamic acid by the enzyme PAL. This product then acts as an intermediate substrate to the enzyme 4CL or C4H at the next entry point of the phenylpropanoid pathway (Rasmussen and Dixon, 1999). The channelling of the intermediates might present a potential biosynthetic flux into two different products, cinnamoyl CoA or 4-coumaroyl CoA. Condensation of these two

products and three molecules of malonyl CoA (the product of acetate from acetyl CoA carboxylase) by CHS forms a chalcone precursor which is further modified into diverse compounds by the enzyme CHI (Dixon, 2005).

In plants, cytochrome P450 monooxygenases are involved in synthesis of diverse metabolites other than phenylpropanoids, including fatty acids, alkaloids as well as terpenoids (Dixon, 2005). The enzyme C4H is a cytochrome P450-dependent monooxygenase of the phenylpropanoid pathway and is responsible for introducing a phenolic hydroxyl group and catalyzing hydroxylation of trans-cinnamate, the central step in the phenylpropanoid pathway (Singh *et al.*, 2009). This enzyme is relatively unstable, low abundance, and membrance-bound (Bell-Lelong *et al*., 1997). Given the importance in many pathways, C4H has been well documented for its' function as well as its regulation. For example, C4H activity is induced by a number of triggers, including light, elicitors and wounding (Russell, 1971; Beneviste *et al*., 1978; Bolwell *et al.*, 1994). Moreover, Lamb and Rubery (1976) and Orr *et al*. (1993) further suggested that the expression of C4H is regulated in response to the application of exogenous phenylpropanoid pathway intermediates such as ρ-coumaric acid. On the other hand, altering enzyme expression *in vivo* by molecular genetic approaches provides a method of studying the enzyme roles without the reliance on exogenous stimuli (Dixon, 2005). Blount *et al.* (2000) genetically modified the expression of C4H and PAL enzyme activity via sense and antisense technology in tobacco plants. However, the C4H enzyme activity was not down-regulated in the PAL knock-down plant. This study has provided an evidence for a feedback loop at the entry point into the phenylpropanoid pathway, in which the regulation was sensed through production of cinnamic acid (the substract of C4H).

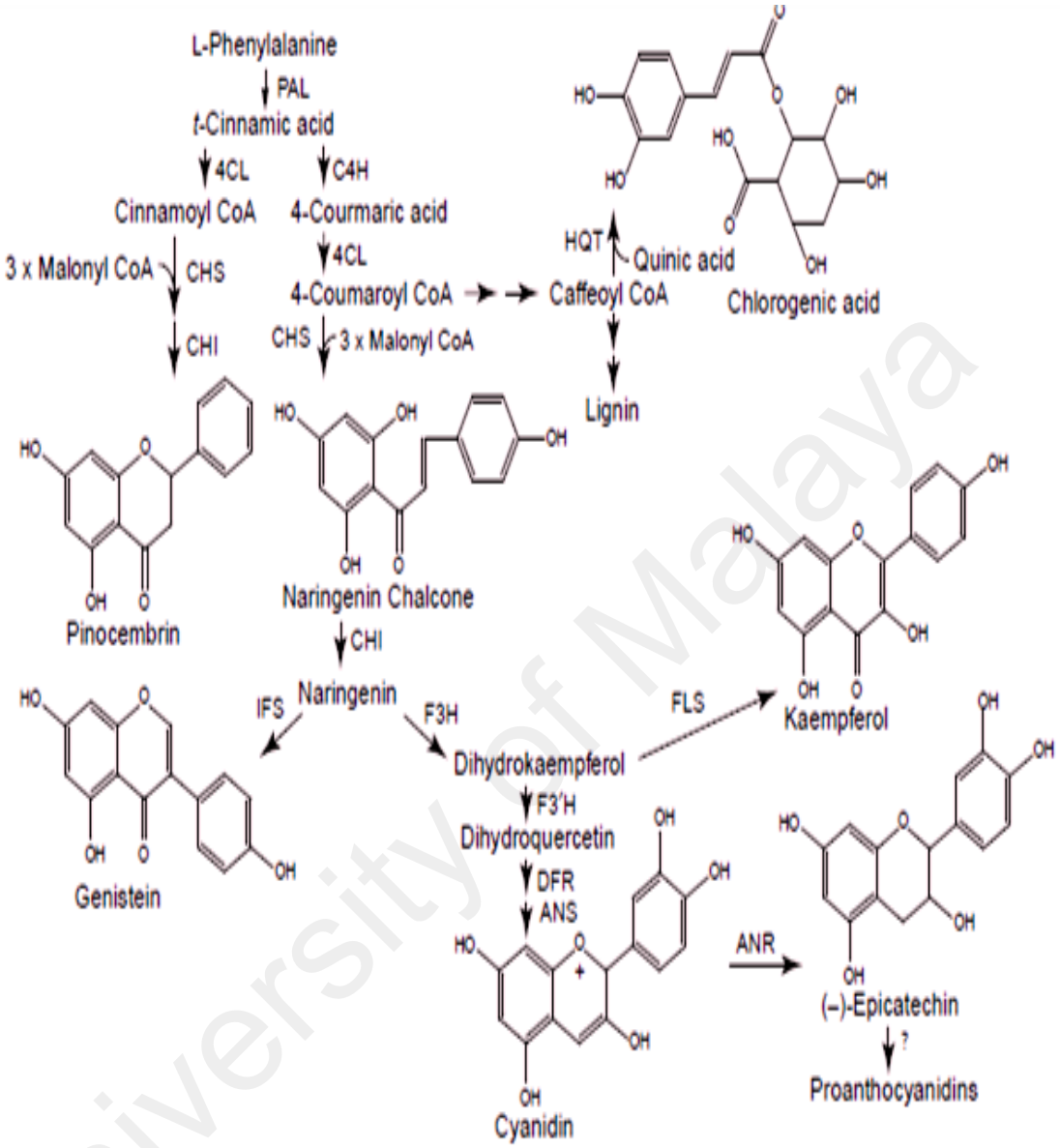


Figure 1.1: Phenylpropanoid pathways in plants.

(Dixon, 2005)

1.5 Plant transformation and genetic engineering of plants

Genetic engineering of plants has a history of more than 30 years, contributing significantly to the challenge in respond to the needs of rapidly growing global population in a sustainable manner and maintaining the environment quality (Liu *et al.*, 2013). Particularly aiming for improvement of crop quality such as yield, herbicide resistance, insect resistance and stress tolerance to adapt to changing and extreme environments which is at utmost importance to food security and maximise the utility of arable land (Collins *et al*., 2008).

Transgenic plants were grown on an estimated 170 million hectares encompass 29 countries including 69.5 million hectares in USA, 36.6 million hectares, 23.9 million hectares in Argentina, 11.6 million hectares in Canada, 10.8 million hectares in India, 4 million hectares in China, 3.4 million hectares in Paraguay, 2.9 million hectares in South Africa, 2.8 million hectares in Pakistan, 1.4 million hectares in Uraguay, 1.0 million hectares in Bolivia and < 1.0 million hectares in Philipines, Australia, Burkina Faso, Myammar, Mexico, Spain, Chile, Colombia, Honduras, Sudan, Portugal, Czech Republic, Cuba, Egypt, Costa Rica, Romania and Slovakia (James, 2012). Bt crops resistance to the bollworm or borer remains the major transgenic crop planted in USA, India and China (James, 2012).

Genetic engineering of plant technology also offers the platform to explore new functions plants capable of, such as biosensing and producing valuable compound (Naqvi *et al.,* 2010). Novel products from non-plant origin such as vaccines and pharmaceuticals can be produced using transgenic plant cell cultures as biofactory (Daniell *et al.*, 2009). Plant-based biofactory when compared to other eukaryotic systems, enable proper post-translational modifications, folding and disulphite bond formation (Yusibov and Rabindran, 2008). It also provides appropriate biological

containment and thus reduced the costs for upstream facility as well as regulatory management (Daniell *et al*., 2009).

Agrobacterium-mediated transformation is the most widely used approach for genetic engineering of plants (Liu *et al.*, 2013). Stable integration of gene(s) in nuclear or organelle genome can be achieved using *Agrobacterium* (Roland, 2014). Other approaches such as biolistic bombardment (Wong *et al.*, 2005), polyethylene glycol treatment of protoplast (Cardi *et al.*, 2010), plant artificial chromosomes (Gaeta *et al.*, 2012), and precise genome editing (Li *et al.*, 2013) also employed for plant genetic engineering purposes.

1.6 RNAi and metabolic engineering in plants

RNA silencing, a phenomenon referred to as posttranscriptional gene silencing (PTGS) in plants (English *et al*., 1996) and RNA interference (RNAi) in animals (Fire *et al*., 1998) is a directed process of homologous messenger RNA degradation (mRNA) which regulates gene expression in a sequence-specific manner. In plants, double-stranded RNA (dsRNA) is crucial precursor, capable of inducing RNA silencing by generating functional small interfering RNAs (siRNAs) with the aid of Dicer-like (DCL) components, counterparts of the Dicer RNase III of animal cells (Molnar *et al.*, 2011).

Biogenesis and function of siRNAs are thought to be conserved in all multicellular eukaryotes that share some similar key components in the RNAi pathway, such as Dicer (RNase-III like dsRNA-specific ribonuclease) and AGO proteins from the Argonaute gene family. In the case of homologous mRNA degradation induced by dsRNA (double-stranded RNA) or hpRNA (hairpin RNA), the mechanism can be divided into two steps: initiation and effector steps (Cerutti, 2003). In the initiation step, 21 to 23 nucleotide (nt) siRNAs are produced from long dsRNA or hpRNA processed by a

Dicer-like complex. Next, the siRNAs will be incorporated into an RNA-induced silencing complex (RISC) which then triggers breakdown of homologous mRNAs using the incorporated siRNA as a guide and result in lower (knockdown) or no (knockout) expression of the targeted mRNA(s) (Lu *et al*., 2004).

RNAi has progressed into a powerful tool for functional genomics, reverse genetics and metabolic engineering studies (Small, 2007). Several approaches have been adopted for efficient delivery of dsRNA or siRNA in plants i.e. hairpin RNA vectors via *Agrobacterium*-mediated transformation, virus-induced gene silencing (VIGS) via virus vectors, and direct synthetic dsRNA induced gene silencing (Sato, 2005). RNAi related phenomena in plants can be traced back in the year of 1986 before the discovery of RNAi by Fire and Mello (2002). One of the earliest phenomena observed was coat protein mediated protection (CPMP) which confers viral resistance to the transgenic tobacco plants by expression of the sense or antisense strand of the tobacco etch virus potyvirus (TEV) coat protein gene sequence (Lindbo and Dougherty, 1992). Co-suppression, which was first observed in transgenic petunia plants is also a RNAi-related phenomenon (Napoli *et al*., 1990). In comparison between dsRNA-induced and antisense-induced RNAi, dsRNA has advantages over antisense technology, in terms of efficiency and stability (Wesley *et al.*, 2001). It also advantages over mutational breeding because of the specificity of silencing in multigene families (Makoto, 2004). Biosynthesis pathway of several groups of secondary metabolites in plants has been manipulated by siRNA-mediated RNA silencing. This includes alkaloids in opium poppy (Allen *et al*., 2007); flavanoids in tobacco (Nishihara *et al*., 2005) and tomato (Schijlen *et al.*, 2007); isoflavones in soybean (Subramanian *et al.*, 2005); anthocyanin in *Torenia hybrid* (Tanaka and Ohmiya, 2008); Benzenoid and phenylpropanoid in petunia (Orlova *et al.*, 2006) and many others. These works not only facilitated the understanding of the biosynthesis pathways of the particular group of secondary

metabolites, but it also helped to identify functional genes and enzymes involved in the biosynthesis pathway. Furthermore, with a clear picture of the biosynthesis machinery, metabolic engineering of valuable compounds in plants becomes feasible.

1.7 The rationale of the study

Amongst the phenylpropanoid products isolated from *B. rotunda*, panduratin A is among the desirable compounds: Tan *et al.*(2005) reported that panduratin A showed the most appreciable inhibitory activities against Dengue 2 viral NS2/3b protease. However, the production of panduratin A is low and limited in nature (Yusuf *et al.*, 2013). Moreover, the complexity of the panduratin A molecule itself has made chemical synthesis of this compound difficult and not economic (Li *et al.*, 2002). Therefore in the current study, it was aimed to introduce a dsRNA from a partial C4H gene sequence of the enzyme, trigger RNAi/ knock-down of the enzyme and examine the RNAi effects on enzyme functions and compound production in *B. rotunda* suspension cell. In addition, development and optimization of an *in vitro* cell culture and *Agrobacterium*-mediated system was also included as the strategy to achieve the aim of the project.

1.8 Objectives of the study

This project aims to:

1. To develop reliable cell suspension culture and *Agrobacterium* – mediated transformation systems for *B. rotunda*

2. To evaluate the knock-down effects of *C4H* gene on the production of secondary metabolite compounds in relation to the phenylpropanoid pathway in *B. rotunda*.

CHAPTER 2: ESTABLISHMENT AND REGENERATION OF *BOESENBERGIA ROTUNDA* SUSPENSION CELL CULTURES

2.1 Introduction

In vitro culture is a key tool of plant biology that exploits the totipotency nature of plant cells, a concept described by Haberlandt (1902) for better understanding of plant physiology, morphology and plant-environment interaction. Stewart *et al.* (1958) demonstrated the first success *in vitro* culture of freely suspended carrot cells capable of regenerating into complete plantlets further mark down the progression in plant tissue culture, which is essential for any crop improvement program as an immediate source of contaminant-free materials (Rao and Ravishankar, 2002).

In vitro cultures are used for genetic engineering via transgenesis or cis-genesis, and also generating genetic variability by producing haploids, somaclonals, mutants and gametoclonal variants for crop improvements (Kothari *et al*., 2010). For example, carrot cultured cells have been a well-defined model for dicotyledonous plant tissue culture studies (Fujimura, 2014). High frequency and synchronous systems in carrots coupled with recent technology such as next generation sequencing has facilitated the studies of dicots embryogenesis developmental biology (Iorrizo *et al*., 2011). Other culture systems such as *Arabidopsis* (Ueda *et al.*, 2011), tobacco (Kim *et al.*, 2003; Wang *et al.*, 2011), cereal like barley and wheat (Harwood, 2012), maize (William *et al.*, 1990), rice (Hiei *et al.*, 1994; Taoka *et al.*, 2011), potato (Yang *et al.*, 2011), tomato (Chetty *et al.*, 2013), banana (Antara *et al.*, 2009; Wong *et al.*, 2005), and *Medicago truncatula* (Iantcheva *et al.*, 2014) are also successfully integrated into plant study and improvements strategies in which often said to be the key to success in realisation of quick and efficient biotechnology advancements (Gamborg, 2002).

2.1.1 Somatic embryogenesis

Somatic embryogenesis (SE), also known as non-zygotic embryogenesis is the developmental process by which somatic cells undergo restructuring and generate into embryogenic cells under suitable induction conditions. These cells then go through a series of morphological and biochemical changes which result in the formation of a somatic embryo and eventually generate into new plants (Schmidt *et al*., 1997; Komamine *et al*., 2005). Somatic embryos resemble zygotic embryos and undergo almost identical developmental stages (Dodeman *et al*., 1997). The observable process and the feasibility to obtain somatic embryos from different types of tissues have allowed them to be used as a model system for morphological, physiological, molecular, and biochemical studies. And also provides a valuable tool for regenerating and propagation with relatively high genetic uniformity (Stasolla and Yeung, 2003).

2.1.1.1 Factors influencing somatic embryogenesis frequency and efficiency

Considerable effort has been expended for better understanding and controlling the process of SE ever since the first observations of somatic embryo formation in carrot cell suspension cultures by Stewards *et al*. (1958). Various external factors including the choice and application of plant growth regulators (PGR) and growth adjuvants, carbon source, light regime, gelling agent, temperature and subculturing regime are influencing SE efficiency (Thorpe, 1995). These external factors are common aspects cell biologist playing around with for optimising SE efficiency and frequency. PGR which generally comprising of five classes: auxins, cytokinins, gibberellins, abscisic acid and ethylene, when use in an appropriate concentration or combination interact with endogenous PGR trigger division or differentiation of the cells. Intermediate ratio of auxin to cytokinin promote vigorous cell division which leads to formation of unorganised mass of cells, while low or high auxin to cytokinin ratio generally promote cell differentiation and

leads to organogenesis (Slater *et al.*, 2003). While SE which involves systematic dedifferentiation usually requires low level of cytokinin and removal of auxin applied during rapid proliferation of embryogenic cells (Fujimura, 2014).

Synchronisation of developing somatic embryo at different phases by removing cells not involved in embryogenesis or non-embryogenic cells also greatly enhances SE frequency and efficiency. Synchronous carrot culture obtained by sieving or Ficoll density centrifugation has enabled determination of phases in carrot SE process which served as a classic reference guide for many (Osuga and Komamine, 1994). Morphology of different phase somatic embryos could be identified and categorised in in four phases as shown in figure 2.1. Embryogenic cell cluster during phase 0 was referred as State 1 cell clusters and progress into State 2 when State 1 cell clusters were transferred into auxin-free medium and proceed into phase 1. Rapid cell division occurs during phase 1 and 2 and ceased when cell differentiate into globular embryo at the final juncture of phase 2 (Komamine *et al.*, 2005). While dicots form heart-shaped somatic embryo and develop into torpedo shaped somatic embryo in phase 3, the monocots do not have an apparent heart-shaped progression but develop into torpedo and form complete plantlets identical to the donor plants.

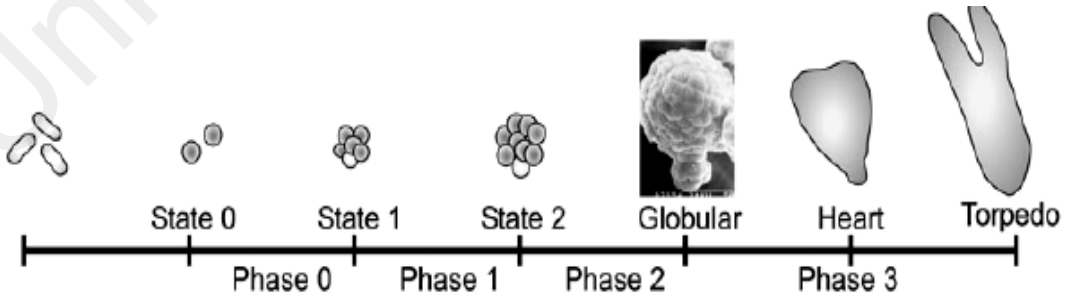


Figure 2.1: Different phases of somatic embryos and their morphology illustration.

(Fujimura, 2014)

2.1.1.2 Molecular regulation of somatic embryogenesis

Somatic embryogenesis (SE) is a process when somatic cells response to chemical and physical stimuli and gain embryogenic competency. Cascade of signals at different level i.e. genomic, gene expression level, proteomics and epi-genetic levels such as miRNA regulating SE (Elhiti *et al.*, 2013; Lakshmanan and Taji, 2000; Reinhart *et al.*, 2002). Gene or group of genes that involved in SE can be classified in three categories according to their developmental stages: embryonic induction, embryonic, and maturation (Elhiti *et al.*, 2013). During embryonic induction, cells dedifferentiate, acquiring totipotency and commit into embryogenesis.

Stress is required for cell differentiation in which proteomics analysis revealed that peroxidase, a stress-related protein was found up-regulated four folds in during SE induction stage of *Medicago truncatula* (Almeida *et al*., 2012) while reverse glycosylation protein and heat shock protein 17 were also found accumulated to high level in early SE of white spruce (Lippert *et al*., 2005). Dedifferentiated cells which are totipotent, potential to develop into a complete adult organism usually characterised by the entirety of their nuclei contain (Gupta and Durzan, 1987). Epigenetic changes caused by chromatin defects also affected totipotency of cells (Birnbaum and Alvarado, 2008). Besides, chromatin remodelling may trigger totipotency genes, such as S*OMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1)* enhanced embryogenic competency in culture (Hecht *et al*., 2001). Several genes also reported to work in parallel or in a sequential order controlling totipotency of somatic cells, such as the transcription factor gene *WUSCHEL (WUS)* (Elhiti *et al.*, 2010), *LEAFY COTYLEDON (LEC1, LEC2)* (Elhiti *et al*., 2012), auxin biosynthetic enzyme genes *YUC2* and *YUC4* (Stone *et al.*, 2008).

Once somatic cells acquire totipotency, cell division is activated and changes the cell fate into meristematic cells from which somatic embryos originated. This embryonic stage of development is mainly controlled by the cyclin-dependent kinases (CDKs). CDKs are complexes of cyclin subunits involved in cell cycle initiation and progression (Zhang *et al.*, 2012). Unequal division of meristematic cells generates polarity and position-dependent cell fate determination (Laux and Jurgens, 1997). Several homeobox genes regulate the cell differentiation especially shoot apical meristem (*STM*) (Sentoku *et al*., 1999), *WUS*, *CLAVATA1 (CLV1)*, *CLAVATA2 (CLV2)* and *CLAVATA3 (CLV3)* (Chen *et al*., 2009) involved in cell fate determination and leading the embryogenic cells towards maturation. During maturation, the cell deposits storage materials, channel storage to appropriate subcellular compartments and obtain adaptation to germination into a complete individual including dessication tolerance as well as certain level of apotosis (Arnold *et al*., 2002). Spatial-controlled apotosis causes the embryo suspensor to detach from the embryo and terminate the dependence of nutrient supply on the suspensor (Bozhkov *et al*., 2005). This programmed cell death marks the transition of somatic embryo into defined orientation comprising of shoot apical meristem and root apical meristem which further develop into a complete plantlet (Fujimura, 2014).

2.1.1.3 Recalcitrant challenge of somatic embryogenesis

Inability of plant tissue cultures to respond to *in vitro* manipulations renders the plant recalcitrant due to genotype factors as well as the time-related reduction and/or loss of morphogenetic competence and totipotent capacity (Benson, 2000; Bonga *et al.*, 2010). This phenomenon can cause difficulties to efficient mass propagation and hinders the development of crops improvement. Some plant species are known for its' recalcitrant nature to SE i.e. *Capsicum chinense* Jacq. (habanero chili) (Avilés-Vinãs *et al.*, 2013; Ochoa-Alejo *et al.*, 2001), coconut palm (*Cocos nucifera* L.) (Verdeil *et al.*, 1994), *Vitis vinifera* (Marsoni *et al.*, 2008), the tea- *Camellia sinenesis* L. (Suganthi *et al*., 2012),

Chinese cotton- *Gossypium hirsutum* L. (Wu *et al.*, 2004), and white spruce trees (Rutledge *et al.*, 2013). Despite the fact that many plant SE models have been employed for better understanding, the mechanism and the cause of recalcitrant remain to be clarified. Investigation of molecular control and regulation of it has been initiated, using cutting edge technology. For instance, a 32 K oligo-probe microarray technology has revealed that SE recalcitrant in *Picea glauca* is related to antagonistic effects from endogenous biotic defence activation (Rutledge *et al.*, 2013). SE recalcitrancy also observed in direct regeneration of *B. rotunda* where percentage of plantlets regeneration from embryogenic callus reduced about half with successive order of subculture (Tan *et al.*, 2005). Therefore, revision of the protocol and efficiency could be carried out using cell suspension culture platform for better performance.

2.1.2 Cell suspension cultures

The use of cell suspension culture for the robust mass propagation of uniform materials is often more appropriate compared to solid cultures, which have limited production capacity (Jayasankar and Litz, 1998). Especially when plant cells are intended for producing useful secondary metabolites and transgenic proteins at high productivity in terms of yield, biomass and ease of scaling-up such as for production of antibodies, vaccines and other biopharmaceuticals (Daniell *et al.*, 2009). Culture growth parameters such as major nutrient, micronutrient elements, PGRs, dissolved oxygen, agitation, temperature and light regime are common factors investigated for significant production venture (Zhao and Verpoorte, 2007). Yield of secondary metabolites from cell suspension cultures is not always proportional to biomass production. To overcome this problem, the cells are treated with an external stimulus, as elicitors (Aharoni and Galili, 2011). Direct contact of the cells with the stimuli in suspension culture enables quick response of the cells when compared to solid cultures. Thus provide a platform for ease of treatment and fast assay for functional analysis (Weathers *et al.*, 2010).

Cell suspension cultures also provide the route for single cell origin genetic transformation with the lowest degree of chimerism (Ghosh *et al*., 2009; Guo and Zhang, 2005). Significant efforts have added advantages and opened the use of cultured cells in genetic manipulation studies which have been incorporated into many breeding programs to provide elite transgenic plants (Jin *et al*., 2005; Li *et al*., 2006). Transgenic plant cell system is superior compared to bacterial or yeast system because plant system is capable of proper post-translational modification, such as protein folding, disulfide bond formation, glycosylation, and lipid modifications (Kim *et al.*, 2003; Daniell *et al.*, 2009). As a result, many biologically active peptides have been produced e.g. human α-interferon in tobacco BY-2 cell suspension cultures (Xu *et al.*, 2007) and Human α 1-antitrypsin in rice cell suspension cultures (Shin *et al.*, 2003). These examples not only demonstrated the usefulness of plant cell suspension cultures, and perhaps most importantly, the advantage of biological containment for transgenic cells in shake-flasks or in bioreactors as the green factory for useful products (Weathers *et al.*, 2010).

2.1.3 Aims of this part of the study

Plantlet regeneration of *B. rotunda* via SE from callus cultures (Tan *et al*. 2005; Yusuf *et al*. 2011a) and direct regeneration (Yusuf *et al*. 2011b) have been demonstrated previously. However, cell suspension culture and its' regeneration protocol has not yet been detailed and optimised. Thus, this part of the study aimed to establish embryogenic cell suspension and SE protocols for application in metabolic engineering via genetic transformation in *B. rotunda* cell suspension culture.

2.2 Materials and methods

2.2.1 Plant materials, explant surface sterilization and callus induction

Fresh rhizomes of *B. rotunda* used in the experiments were supplied from a commercial farm in Termerloh, Pahang, Malaysia. Rhizomes obtained from the field were thoroughly cleaned with tap water and soap and kept in a black plastic bag for sprouting. Buds of about 1 – 2 cm in length were cut from the rhizomes for surface sterilization. Sliced meristems were then placed on media supplemented with 1.0 mg/L α- Napthaleneacetic Acid (NAA) and 1.0 mg/L of 6-Benzyladenine (6-BA), 1.0 mg/L Indole-3-acetic acid (IAA), 30 g/L sucrose and 2 g/L Gelrite® (Sigma, US) for callus induction as described by Tan *et al* (2006). Calli were then propagated in MS media supplemented with 3 mg/L (2,4-dichlorophenoxy)acetic acid (2, 4 – D) and 2 g/L Gelrite® (Sigma, US). The pH of the medium was adjusted to 5.8 with hydrochloric acid (HCl) and sodium hydroxide (NaOH) prior to autoclaving at 121 ºC for 20 minutes. All cultures were prepared under aseptic conditions and grown at 26 ºC under 16 hours light/ 8 hours dark photoperiod with a light intensity of 31.4 µmol $m^{-2}s^{-1}$ provided by cool fluorescent lamps.

2.2.2 Suspension initiation, maintenance and propagation

For suspension cell culture initiation, one clump of callus (≈0.5g) was inoculated in 50ml MS basal Liquid Media (LM) supplemented with 1mg/L 2, 4-D, 100mg/L L-glutamine and 20g/L sucrose in 250ml Schott Duran® Erlenmeyer flasks and cultured on a rotary shaker at 80 rpm. The pH of LM was adjusted to 5.7 prior to autoclaving at 121 ^{o}C for 20 mins. Suspension cultures were maintained and subcultured every 14 days with replacement of the media at a ratio of 1 to 4 (old media to new media). Suspension cell from a flask was divided into 2 or 3 new flasks depending on the amount of cell harvested. Ten ml of old media with cells was inoculated into 40ml new media and cultured at 26^{o}C in the growth room under 16 hr photoperiod. Cells were sieved through

a nylon filter sized 425 µm to obtain small cell clumps. Settled cell volume (SCV) of the suspension culture was measured and the growth was recorded according to Wong *et al*. (2013). The data was recorded from 3 biological samples based on 5 experiments.

2.2.3 Regeneration of suspension cell culture

For regeneration, cells were spread onto Whatman® no. 1 filter paper which had been placed on PGR-free media (MS0) supplemented with 30 g/L sucrose and 2.0 g/L Gelrite® (Sigma, US). Plates were then kept in the dark in a growth room at 25 ± 2ºC in the dark. Somatic embryos were counted and subsequently transferred onto media supplemented with different concentrations of NAA and 6-BA for germination, elongation and rooting. Microscopic observation of the somatic embryos was carried out using a Zeiss Stemi SV C stereomicroscope equipped with a MicroPublisher 5.0 RTV camera (Qimaging, Canada), Gel-Pro ® Analyzer (MediaCybernetics, USA). Statistical analysis was performed using ANOVA (SPSS, Inc, US) with Duncan's multiple comparison test at a 95% confidence level.

2.2.4 Histology and microscopic examination

Histological slides of the somatic embryos and cells were prepared using resin fixed in glutaraldehyde-paraformaldehyde-caffeine (GPC) fixative solution (0.1 M phosphate buffer, pH 7.2, 2% (v/v) paraformaldehyde, 1% (v/v) glutaraldehyde, and 1% (w/v) caffeine), dehydrated in ascending ethanol concentration (50%, 70% and 90%), infiltrated and embedded into historesin (Leica Historesin Embedding Kit). Fully polymerized resin was sectioned at 3 µm using a microtome. Sections were stained with 1% periodic acid for 5 min, Schiff's reagent for 20 min and counterstained with Naphtol blue black at 60ºC for 5 min (Yusuf *et al.*, 2011). Slides were examined using an Axiovert 10 inverted microscope (Zeiss, Germany) equipped with a MicroPublisher 5.0 RTV camera (Qimaging, Canada) and Gel-Pro® Analyzer (MediaCybernetics, US).

2.3 Results and Discussion

2.3.1 Callus initiation, suspension cell cultures establishment

Two types of callus were obtained as shown in figure 2.2.The first type of callus (Type I: Fig 2.1a) was friable, yellowish in colour and uniform size with rounded edge, while the second type was compact, whitish, pale in colour and varied in size with an irregular surface (Type II: Fig 2.2b). Type I callus, which produced embryogenic cell mass after 6-8 weeks of culture was selected for establishing cell suspension cultures. Pre-embryogenic masses (PEM) formed on top of the calli (Figs. 2.2c & 2.2d) were selected under a microscope for initiating suspension cultures. A fine, homogenous suspension culture was obtained after two months of regular subculturing and sieving through 425 µm nylon mesh (Fig. 2.2e). The growth of the cell suspension was recorded by measuring the settled cell volume (SCV) of the cultures (Appendix A). The cell cultures started the exponential growth after 5 days of culture and were at the stationary phase after day 20. The cultures showed a stable sigmoidal growth curve with eight-fold increase in SCV (maximum 4.0 ml SCV) after 20 days of culture with a starting inoculum of 0.5 ml settled cells (Fig. 2.3). Histological examination (Fig. 2.2f) showed the suspensions to be composed of spherical cells with similar morphology in small aggregates. Dense cytoplasmic cells with intense nuclei gave an early indication of embryogenic character of the suspension cells. The population of vacuolated and elongated cells was less than the dense cytoplasmic cells (Fig. 2.2f).

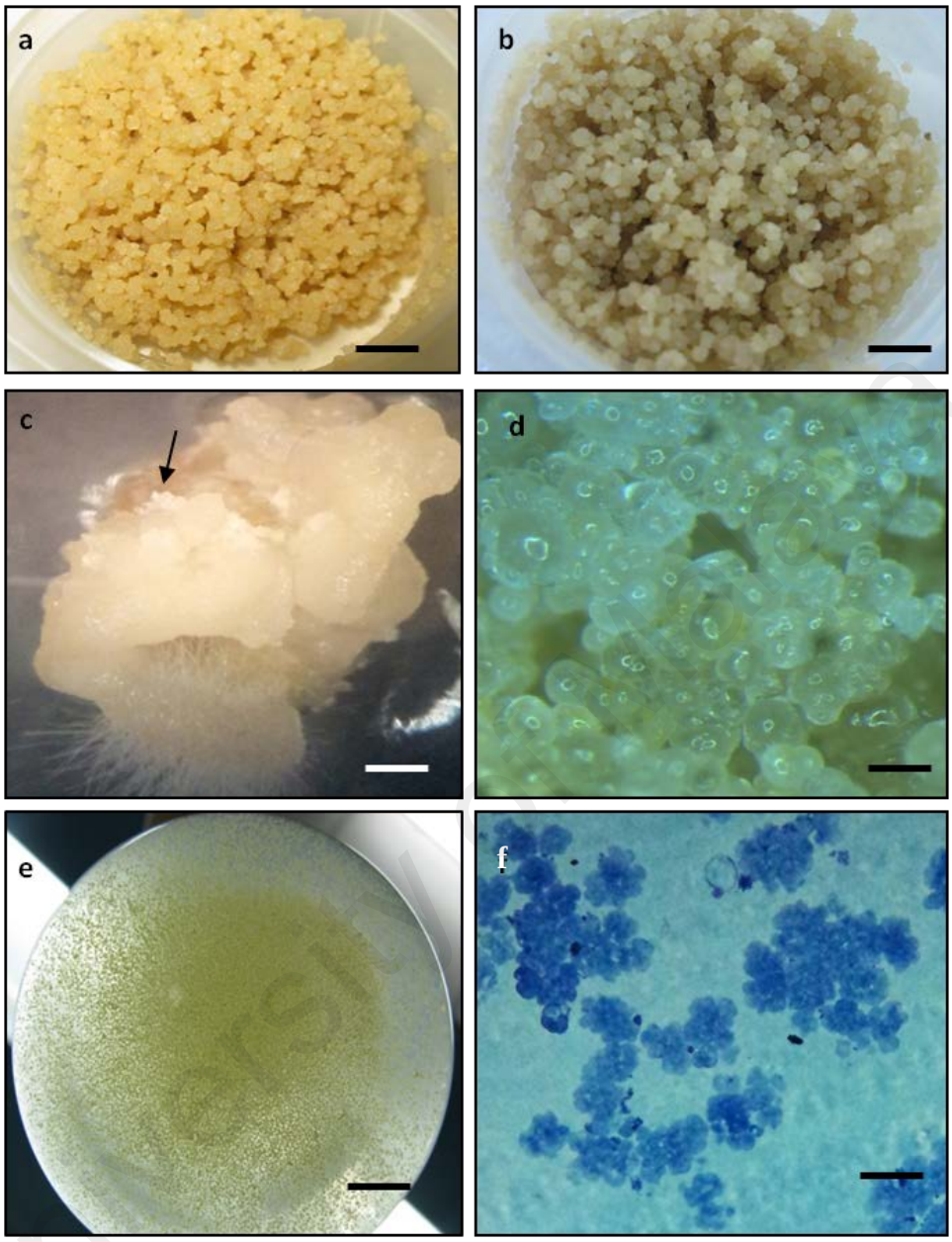


Figure 2.2: Different types of callus obtained. (a). Type I callus, bar = 1 cm. (b). Type II callus, bar = 1 cm. (c). Swollen explants with PEM (indicated by arrow) formed on top, bar = 1.0 mm. (d). PEM, bar = 100 µm. (e). Fine and homogenous cell suspension obtained after sieving, bar = 1 cm. (f). Histological sections of suspension cells, bar =10 µm.

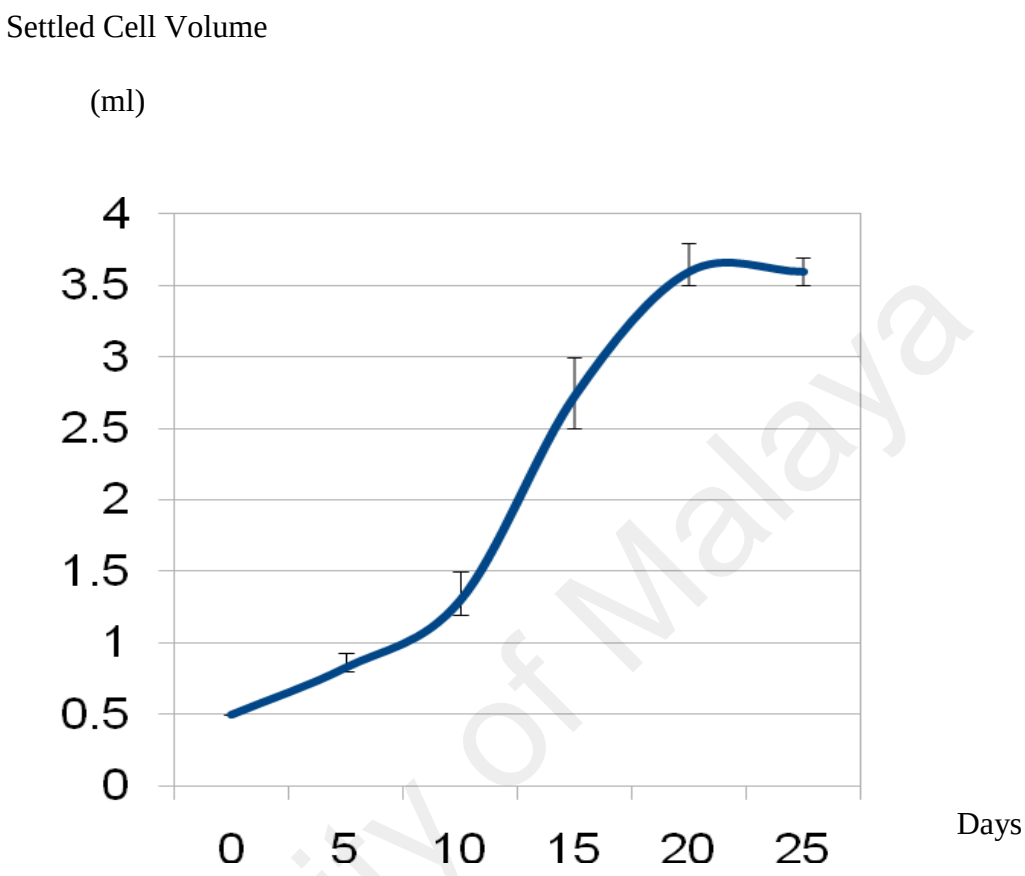


Figure 2.3: The growth of the fine, embryogenic suspension cell culture. Data were recorded based on 3 biological replicates and 5 technical replicates.

2.3.2 Regeneration of *B. rotunda* cell suspension through somatic embryogenesis

Embryogenic masses (EM) were first observed after four weeks after transferring onto solid MS0 media. Translucent somatic embryos (Fig. 2.4a) were counted under the microscope and data were taken six weeks after plating. Healthy SE started to develop into mature embryos 6 to 8 weeks after plating (Fig. 2.4b). The result showed that MSO was sufficient for regenerating somatic embryos from cell suspensions plated without any supplement of phytohormone. Moreover, cell browning occurred when cells were plated on media supplemented with a low level of 2, 4-D (0.5 mgL^{-1}) (Fig. 2.4c).

2.3.2.1 Effects of different inoculation volumes on regeneration

The number of SE developed was influenced by different inoculation volumes (SCV) plated (Fig. 2.5). The highest number of SE was obtained when 50 µl SCV of cells were used as inocula, with an average number of 1433.33 ± 384.41 SE developed per ml SCV. The number of SE formed decreased when the SCV plated was increased. Only 354.88 ± 200.04/ ml SE developed when 100 µl SCV of the cell was used as inoculum. The result is in accordance to Toshihiro *et al.* (1999) where an inhibition effect on SE formation in high cell density embryogenic cell cultures of carrots was found. Amount of inoculated population density of suspension cell cultures was found to be important for SE in some studies (Ibaraki 2001; Koichi *et al*. 1997; Vengadesan and Pijut, 2009). The presence of soluble signaling molecules and interacting factors secreted by cells in conditioned liquid media was observed to promote differentiation in embryogenic cells. Extracellular protein, such as a variety of endochitinase, arabinogalactan and lipochitooligosaccharides which stimulate the development of SE, was found to be cell-density dependent. High cell density is likely to produce more of these proteins and interacting factors which can be inhibitory to SE (Feher, 2005).

Figure 2.4: Embryogenic mass developed from suspension cell. (a) EM developed on PGR-free media. Bar = 200 µm. (b). Mature embryos. Bar = 1 mm (c). Cell browning occurs on plate supplemented with 2,4-D. Bar = 5 mm.

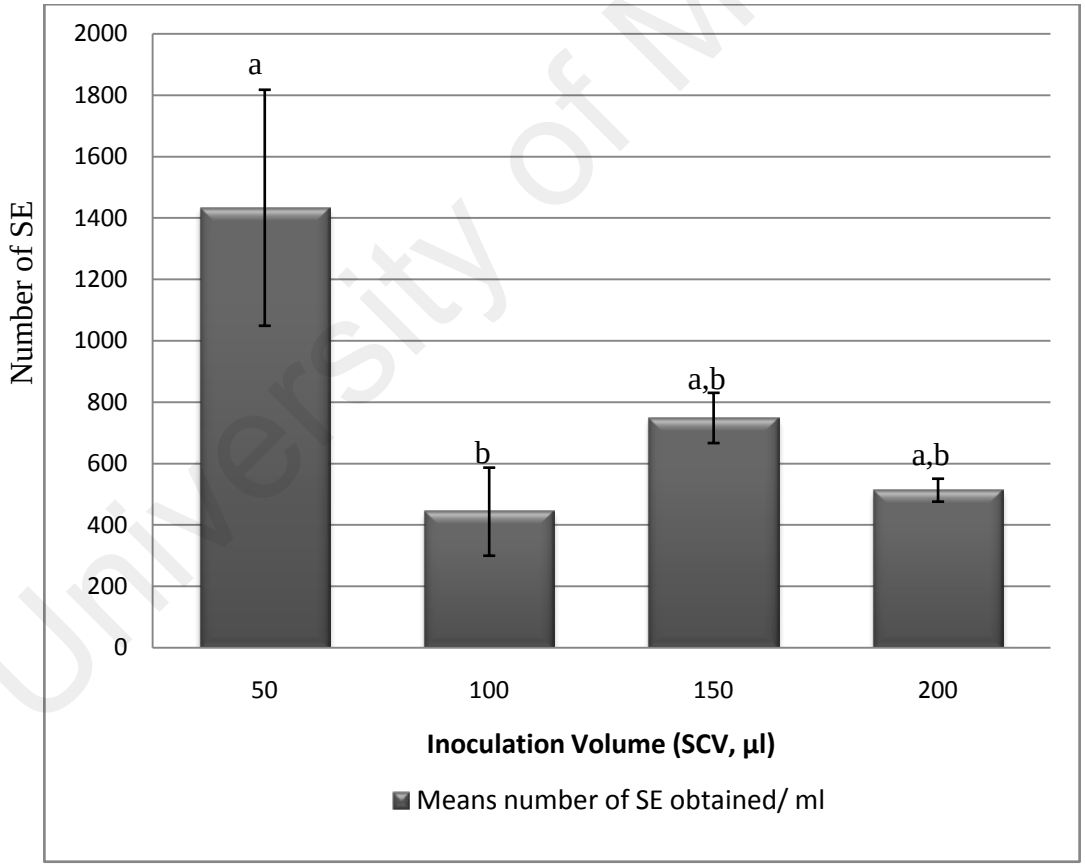


Figure 2.5: Effect of different inoculation volumes on the number of somatic embryos developed on hormone free MS media. Data are means ± SE where n = 3. Different letters indicate significant differences at 95% by Duncan's multiple comparison test. Low cells inoculation volume (50 µl) resulted in the highest number of somatic embryos formation.

2.3.2.2 Germination and development of somatic embryogenesis

White-coloured mature embryos or embryoid structures (Fig. 2.6a) were observed about one week after SE development and transferred to germination media supplemented with various concentrations of 6-BA and NAA. Whitish primordial shoots (Fig. 2.6b) started to be seen 1-2 weeks after the transfer. Some of the coleoptiles of the primordial shoots started to unfurl as early as in the first week of transfer (Fig. 2.6c). However, data were collected after four weeks of transfer for standardisation purpose. The percentage of shoot-forming embryoid structures is shown in Fig. 2.7. A percentage of 16.2 ± 6.4 embryoids germinated and developed on hormone–free MS0 media. The highest number of shoots formed on media supplemented with 3 mg L^{-1} 6-BA and 1 mg L^{-1} NAA with a percentage of 53.5 ± 7.9, equal to approximately 770 plantlets /ml SCV plated on MS0. This frequency appears to be promising when compared to plantlet regeneration via SE on solid media (Tan *et al*. 2005). When media supplemented with 6-BA alone was used, 27.3 ± 6.0% of explants were germinated and developed into complete plantlets in media with 2 mg L^{-1} 6-BA. The number decreased when the concentration was elevated to 3 mg L^{-1} 6-BA but however increased when NAA was added in a lower ratio. This suggested a synergistic effect between 6-BA and NAA on the germination and development of the embryoids of *B. rotunda*. Explants which did not germinate during the observation period, dedifferentiated to form morphogenic callus with further subculture on the same media composition, forming shoots eventually. All regenerants rooted simultaneously and turned green when cultured under 16 hr photoperiod. A successfully acclimatised plantlet with well-developed roots and maroon leaf sheaths, a distinct feature of *B. rotunda* plant, is shown in Fig. 2.6d. All plantlets showed normal *ex vitro* growth after transferring to soil.

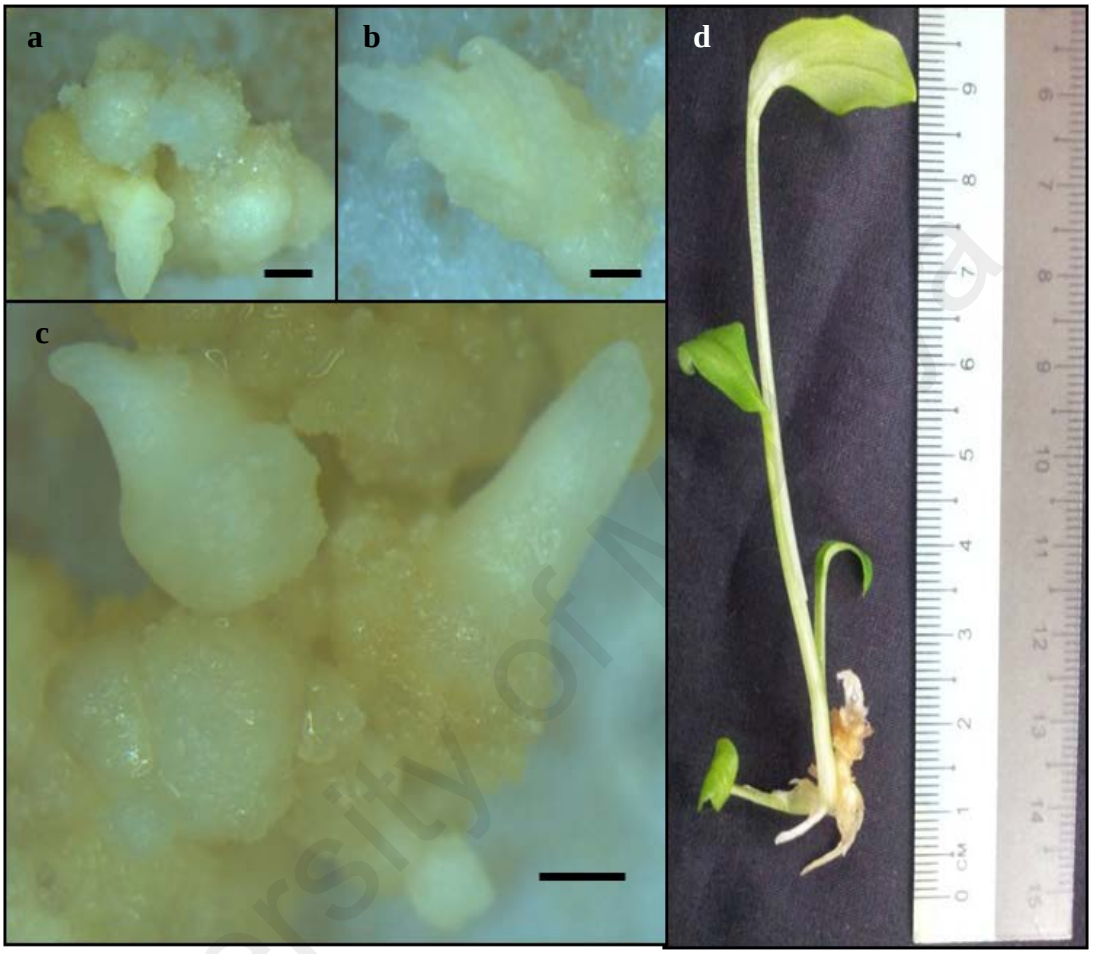


Figure 2.6: Germination and development of *B. rotunda* somatic embryo stages: (a) Matured embryo, bar = 1 mm (b) Developed coleoptiles of primordial shoot, 1 mm (c) Primordial shoots, bar = 1 mm (d) Healthy plantlet regenerated from cell suspension.

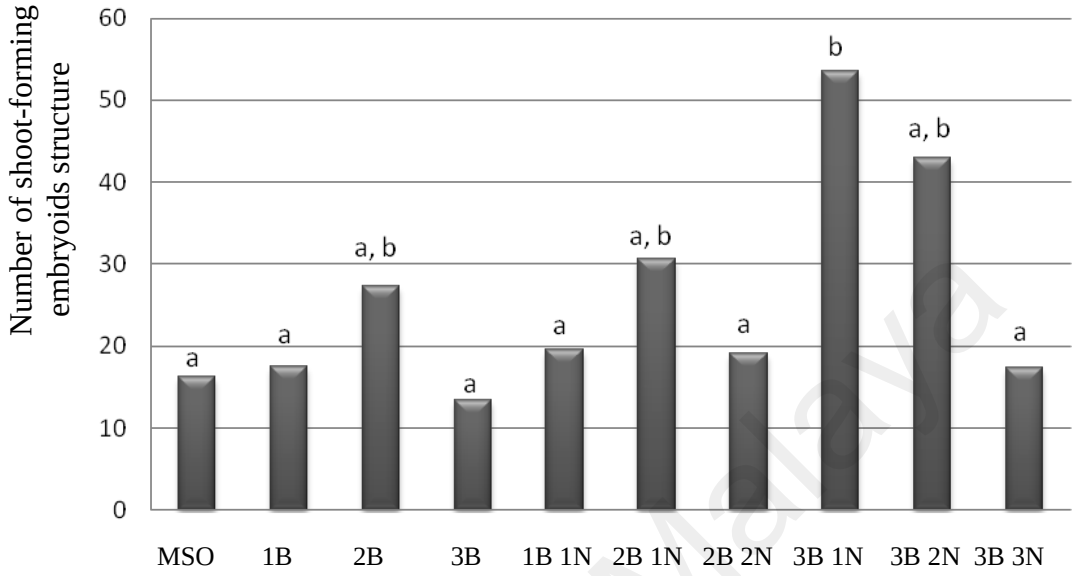


Figure 2.7: Frequency of shoot(s)-forming embryoids germinated and developed on media supplemented with various concentrations of NAA and BA. Error bars represent SE. Different letters indicate significant differences at 95% by Duncan's multiple comparison test.

MS0 = MS media without PGR;
1B = MS with 1 mgL^{-1} 6-BA;
2B = MS with 2 mgL^{-1} 6-BA;
3B = MS with 3 mgL^{-1} 6-BA;
1B1N = MS with 1 mgL^{-1} 6-BA and 1 mgL^{-1} NAA;
2B1N = MS with 2 mgL^{-1} 6-BA and 1 mgL^{-1} NAA;
2B2N = MS with 2 mgL^{-1} 6-BA and 2 mgL^{-1} NAA;
3B1N = MS with 3 mgL^{-1} 6-BA and 1 mgL^{-1} NAA;
3B2N = MS with 3 mgL^{-1} 6-BA and 2 mgL^{-1} NAA;
3B3N = MS with 3 mgL^{-1} 6-BA and 3 mgL^{-1} NAA.

CHAPTER 3: GENETIC TRANSFORMATION OF *B. ROTUNDA* CELL SUSPENSION CULTURES

3.1 Introduction

3.1.1 *Agrobacterium* and plant transformation

Indirect gene transfer to plants methods are based on the utilisation of *Agrobacterium*, a soil borne, gram-negative bacterium which is a natural pathogen to dicotyledonous plants. The pathogenicity of *Agrobacterium* to plants varies depending on the species of bacteria and host. *A. tumefacies* causes "crown gall" disease in plants (Smith and Townsend, 1907), while *A. rhizogenes* causes "hairy roots" (White and Nester, 1980). *Agrobacterium*-mediated transformation has been successfully reported for more than 120 species from at least 35 families including crops of economic importance, vegetables, herbs, fruits, tree, pasture plants as well as ornamental plants (Birch, 1997).

Efficient methodologies have been established for *Agrobacterium*-mediated transformation in dicotyledonous plants which are the natural host range for *Agrobacterium*. In addition, a number of monocotyledonous plants including rice (Hiei *et al*., 1994; Cheng *et al*., 1998) wheat (Cheng *et al*., 1997), maize (Ishida *et al*., 1996), sorghum (Zhao *et al*., 2000) and sugarcane (Enríquez-Obregón *et al*., 1997) have been transformed with *Agrobacterium*. Moreover, with the advancement of vector construction and modification, early problems faced during *Agrobacterium* transformation of monocotyledonous plant cells have been reduced (Liu *et al*., 2015; Rustagi *et al*., 2015).

3.1.2 Ti plasmid and T – DNA of *A. tumefaciens*

The plant transformation ability of *A. tumefaciens* lies in the ability to introduce a segment of its tumour-inducing (Ti) plasmid (Hooykaas and Schilperoott, 1992), the transferred DNA (T-DNA) into the plant nuclei where it becomes integrated into the genome of the host plant (Grant *et al.*, 1991). The Ti plasmid of *A. tumefaciens* is a relatively large plasmid of approximately 200 kilo basepairs (kb). *Agrobacteria* are classified according to opines such as mannopine, agropine and fructopine which are the metabolic substrates produced by the host plant required by the *Agrobacterium* (de la Riva *et al*., 1998). The genes for the production of opine are present inside the T-DNA region of wild type Ti-plasmids. Other than the opine synthesis genes, the oncogenic genes also reside inside the T-DNA region of Ti plasmids. Integration of T-DNA borne oncogenes into a plant genomes will result in crown-gall formation as a consequence of higher exogenous levels of plant growth regulators (PGR), auxin and cytokinin. These PGR stimulate cell divisions that lead to tumour formation.

The T–DNA is flanked by a left border (LB) and a right border (RB) of 25 bp imperfect direct repeat sequences. The consensus sequences of the T–DNA borders for nopaline strains and octopine strains Ti plasmids are shown as in Fig. 3.1. The LB and RB border sequence is crucial and determines the T–DNA transfer in a polar fashion (Wang *et al.*, 1984). Abolishing the first 6 bp or the last 10 bp of the T–DNA border sequence blocks T–DNA transfer (Wang *et al.*, 1987). Moreover, these direct repeats also act as a cis element or enhancer at the right border (Peralta and Ream, 1985). Outside the T–DNA region, resides the origin of replication, conjugative transfer region, the virulence (*vir*) genes and the genes that encode the enzymes for opine catabolism. The opine catabolism genes are transcribed by the crown gall cells, producing enzymes that are vital for *Agrobacterium* to utilize opine as a source of carbon and nitrogen (Hooykaas and Schilperoort, 1992).

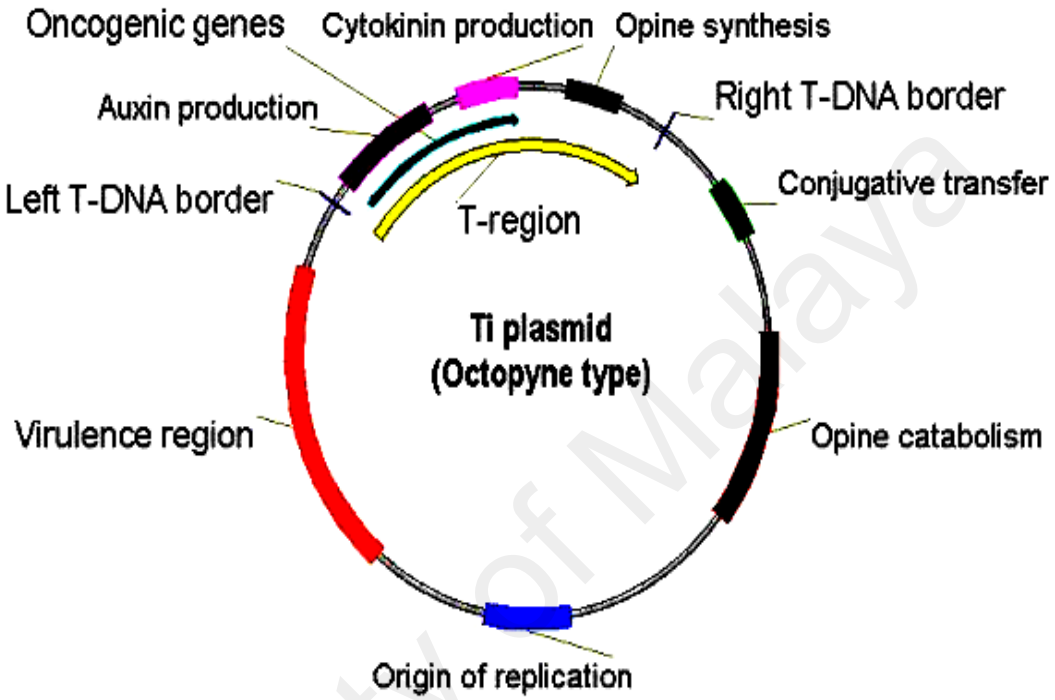


Figure 3.1: General Ti – plasmid map.

(Hoekema *et al*., 1983)

3.1.3 The T – DNA transferring machinery and mechanism

The process of T-DNA transfer involves three genetic elements: one chromosomal element, the chromosomal virulence genes (*chv*), and two elements from the Ti-plasmid itself, the LB and RB, and the Ti plasmid virulence genes (*vir*). The vir genes on the Ti plasmid derive from six operons (*virA, virB, virC, virD, virE* and *virG*) and play important roles in transferring T–DNA (Hooykaas and Schiilperoort, 1992; Zupan and Zambryski, 1995). The *virA, virB, virD,* and *virG* are necessary for T-DNA transfer whilst virC and virE function in transferring efficiency. Hence, tumour formation is suppressed in strains with mutations in *virC* and *virE* genes (Draper and Scott, 1991). The only constitutive operons, *virA* and *virG* coding the products *VirA* and *VirG* are of importance in activating the transcription of the other vir genes.

The chv loci (*chvA, chvB* and *chvE*) play important roles in attachment of the bacteria to plant cells (Cangelosi *et al*., 1987). The *chvA* and *chvB* loci are involved in the synthesis and excretion of β-1, 2 glucan that acts as adhesive or signaling molecules in the attachment of bacteria to the plant cells (Cangelosi *et al.*, 1989). Meanwhile, chvE showed its functional role in bacterial chemotaxis and vir genes induction (Ankenbauer *et al*., 1990). The process of T-DNA transfer involved several essential steps: (1). Bacteria colonisation; (2) *vir* genes induction; (3) T-DNA complex transfer; (4) T-DNA integration (Subramoni *et al*., 2014; de la Riva *et al*., 1998) as illustrated in figure 3.3.

Bacterial colonisation takes place when the *Agrobacterium* attach on the plant cell surface with the aid of polysaccharide on the *Agrobacterium* cell surface (Bradley *et al*., 1997). This polysaccharide appears to be the product of the *Agrobacterium* chromosomal 20 kb att locus (Thomashow *et al*., 1987). When the *Agrobacterium* perceives signals such as phenolics and sugars being released by the wounded plant cells, the *vir* genes operons (*virB, virC, virD* and *virE*) are co-ordinately activated by

VirA-VirG components when VirA autophophorylates itself and further phophorylates the *virG* product (Galun and Breiman, 1998).

The activation of *vir* genes operons generates single-stranded (ss) molecules of the bottom strand of T–DNA by nicking upon recognition of the T–DNA LB and RB borders by the proteins VirD1 and VirD2 (Zupan and Zambryski, 1995). VirD2 protein remains covalently attached to 5'-end of the ss T-DNA and protects it from exonucleolytic degradation and distinguishes them as the leading end of T-DNA transfer complex (Dürrenberger *et al*., 1989). The ss T-DNA-vir D2 complex is exported from the bacterial cell by a 'T-pilus' composed of proteins encoded by the *virB* operon and virD4 (Dandekar and Fisk, 2005). In the meantime, VirC1 protein repairs and synthesises the displaced strand (Scheppler *et al*., 2000). Once inside the plant cytoplasm, the virE2 proteins cover the ss T-DNA, facilitates nuclear localisation and leads T-DNA-VirD2 complex to passage through the nuclear pore complex (NPC) in correct confirmation (Citovsky *et al*., 1992; Zupan *et al*., 1995).

The nuclear localisation signal (NLS) of VirD2 and VirE2 direct the T-DNA towards plant cell chromatin (Bravo Angel *et al*., 1998) and promotes integration by illegitimate recombination (Gheysen *et al*., 1991). Once integrated, repair mechanism of the plant cell will be activated for its own DNA (Puchta, 1998).

3.1.4 The pCAMBIA vectors and the reporter systems

The pCAMBIA vector is a derivative of the pPZP family of *Agrobacterium* binary vectors (Hajdukiewicz *et al*., 1994). The vectors offer several advantageous features as they contain a wide range of unique restriction sites for advanced construction, produce high copy numbers in *E. coli* and stable replication in *Agrobacterium*, and carry convenient bacterial and plant selection marker genes.

pCAMBIA1304 (Fig. 3.2) is 12361 bp in size, containing a hygromycin (*hyg*) resistance gene at the LB of the transferred region (Hajdukiewicz *et al*., 1994). Since the RB leads first during the in T-DNA transfer process, hygromycin resistance is present only when the passenger gene is obtained by the plant cell. Besides, it possesses an mgfp5:gusA fusion as a reporter and kanamycin resistance for bacterial selection. Reporter genes are crucial elements in plant transformation vectors, as a means of assessing gene expression and as easily scored indicators of transformation. Sometimes, they are used in place of selectable markers (Slater, 2003). Besides, they are useful tools for the study and analysis of regulatory elements (Thomas *et al*., 1990). However, only a small number of reporter genes are in widespread use, these being β – glucuronidase (*uidA* or *gus*) from *E. coli* (Jefferson *et al.*, 1987), green fluorescent protein (*gfp*) from the jellyfish, *Aequorea victoria* (Haseloff *et al.*, 1997), luciferase genes (*luc*) from the firefly *Photinus pyralis* (Ow *et al.*, 1986), luciferase from the marine bacterium *Vibrio harveyi* (*luxA* and *luxB*) (Koncz *et al.*, 1987) and the chloramphenicol acethyltransferase gene (*cat*) from *E. coli*.

Reporter genes are important for establishment of optimal conditions for transformation. Particularly in the case of *Agrobacterium*-mediated transformation, complex processes are involved and many aspects of the mechanisms still remain unknown. Nevertheless, de la Riva *et al*. (1998) explained the mechanism and the machineries involved as shown in Fig. 3.3.

The β – glucuronidase gene is one of the most widely used reporter genes in plant transformation vectors. The product of this gene (GUS) is a hydrolase that catalyses the cleavage of a variety of β – glucoronides. It can be assayed easily, quickly without involving radioactive methods (Jefferson *et al*., 1987). Quantitative data can be obtained utilising fluorogenic substrates such as 4-methylumbelliferry-β-D-glucuronide (4-

MUG). Meanwhile, the chromogenic substrate 5-bromo-4-chloro-3-β-D-glucuronide (X-Gluc) is used in histochemical staining assays to obtain qualitative results. Besides, it has an advantage because there is little or no GUS endogenous activity in most plant cell.

The green fluorescent protein (GFP) was originally isolated from the bioluminescent jellyfish *Aequorea victoria* and emits bright green light that is proportional to the amount of protein present upon excitation with long-wavelength ultraviolet (uv) or blue light (Morise *et al*., 1974). Its intrinsic, cell-autonomous fluorophore forms autocatalytically without any requirement or substance except for oxygen (Cody *et al*., 1993). It finds immense applications in every field of biological sciences, especially in genetic engineering of plants. It allows direct visualisation of gene expression in living cells without the need for invasive methods and addition of toxic substrates. Thus, it serves as a continuous "real–time" screenable marker for transgene expression in transgenic plant cells (Chalfie *et al*., 1994).

GFP has been widely used as a non-destructive reporter system for both monocots and dicots (Elliot *et al*., 1998, 1999). Niedz *et al*. (1995) reported the first transgenic plant with inserted jellyfish gfp gene. The group demonstrated successful expression of GFP protein in *Citrus sinensis* protoplasts. Though, some reported poor or no fluorescence in Arabidopsis cells and plants transformed with the wild type gfp gene (Haseloff and Amos, 1995; Hu and Cheng, 1995; Sheen *et al.,* 1995). This setback has been prevailed over with the detection of an aberrant mRNA splicing of *gfp* gene in Arabidopsis. A cryptic intron was then removed by altering the codon usage of *gfp* gene using oligonucleotide-directed mutagenesis to avoid mis-splicing in Arabidopsis plants. Bright fluorescence was then achieved in Arabidopsis plant with proper expression. This modified gene, *mgfp4* was then fused to endoplasm reticulum (ER)

targeting peptides to circumvent difficulty in regenerating fertile transgenic Arabidopsis plants. Subcellular localisation of the GFP protein had solved the problem wherein accumulation of free radicals generated upon excitation in cytoplasm was toxic to plant cells (Haseloff *et al*., 1997). Subcellular localisation of GFP proteins was found to be useful as a marker or tracer for studying recombinant proteins compartmentation in vivo (Rizzuto *et al*., 1995) as well as native proteins transportation along secretory pathway.

To meet the demand for better reporter genes for plant transformation, more variants of *gfp* were developed. These variants served the purpose better with enhanced, brighter fluorescence, increased solubility in cytoplasm (Davis and Vierstra, 1998), better temperature stability (Siemering *et al*., 1996), and shifted excitation and emission spectra (Kato *et al*., 2002).

Most of these improved versions of GFP were generated using site-directed mutagenesis methods. Other than this, new fluorescent proteins isolated from different species were also exploited in plant transformation experiments. This included the red fluorescent protein (DsRed) from tropical corals (Clontech Laboratories, California) which was first used in *Agrobacterium*-mediated transformation of tobacco mesophyll cells (Kato *et al*., 2002).

3.2 Specific objective of this part of the study

This part of the study aimed to optimize the *Agrobacterium*-mediated transformation system for cell suspension culture of *B. rotunda* for application in RNAi of C4H.

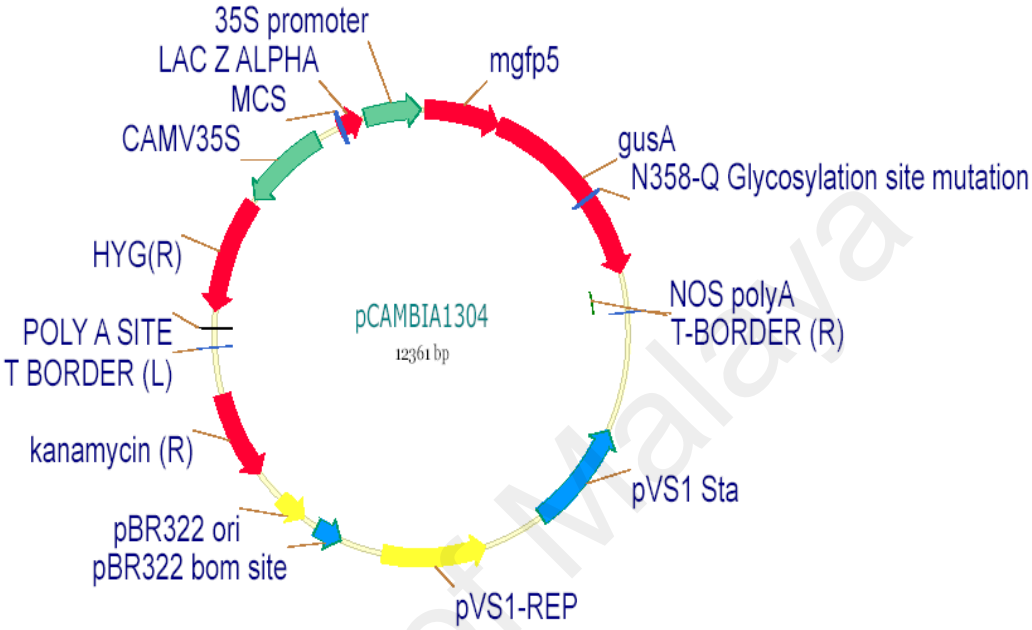


Figure 3.2: The pCAMBIA1304 vector

(Hajdukiewicz *et al*., 1994)

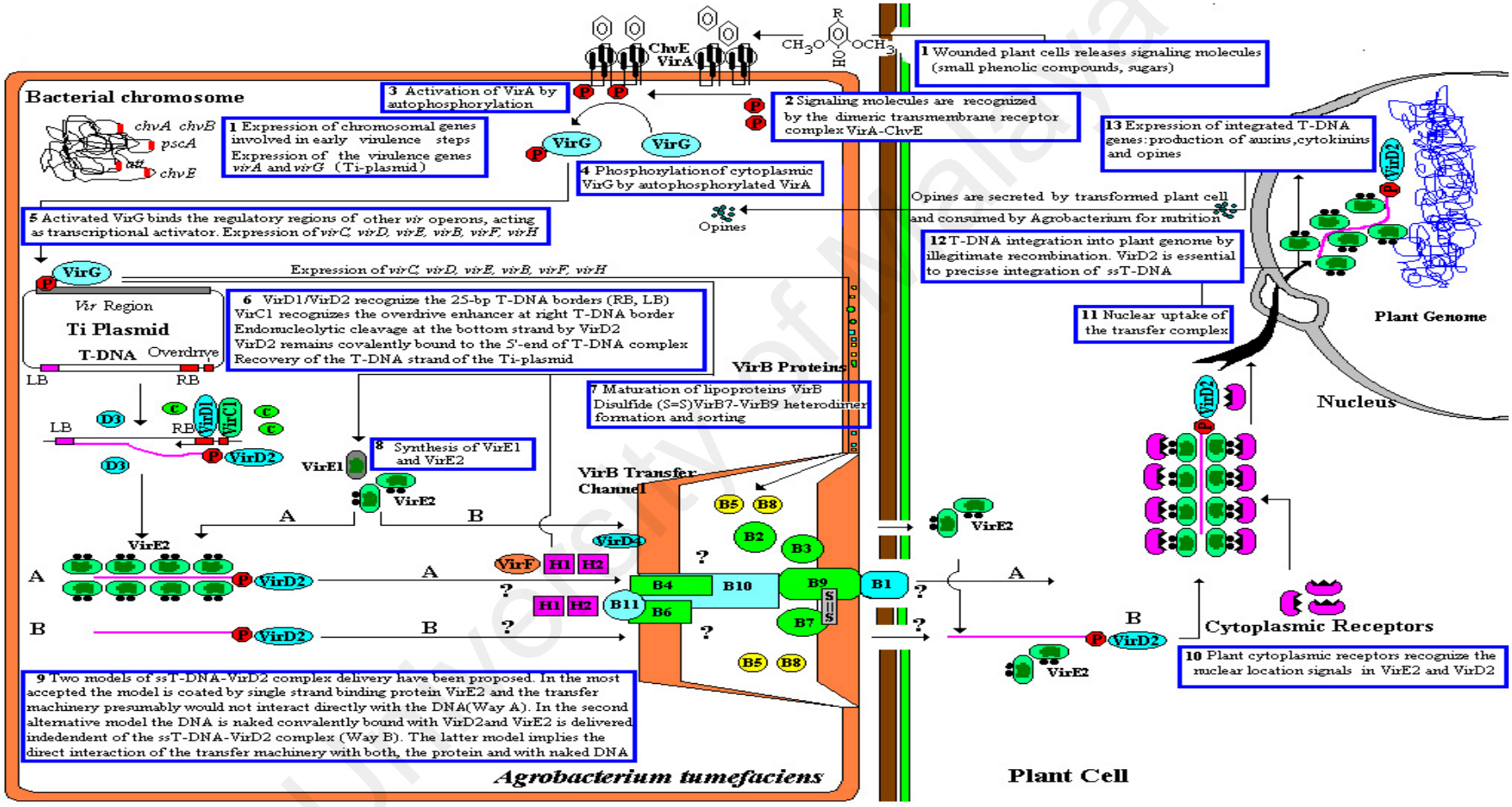


Figure 3.3: *Agrobacterium*- mediated gene transferring mechanisms. Source: de la Riva *et al.*, (1998).

3.3 Materials and Methods

3.3.1 Minimal inhibitory concentration (MIC) of *B. rotunda* suspension cells

This experiment was carried out to determine the minimal inhibitory concentration of the antibiotic hygromycin for effective negative-selection of transformed *B. rotunda* suspension cultures. Suspension cultures were exposed to different concentrations of hygromycin incorporated in liquid media and cultured under standard conditions as described in Section 2.2.3. Growth/ inhibition of the suspension cultures were observed by measuring SCV of the suspension cultures.

3.3.2 *Agrobacterium* – mediated transformation

B. rotunda suspension cultures were used as target tissues for transformation experiments. The *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al*., 1983) harbouring a binary vector pCAMBIA1304 (Cambia, Australia) was used in transforming *B. rotunda* suspension cells. The plasmid carries a *nptII* and a *hpt* genes for bacterial and plant antibiotic selection, and a *gus* and *mgfp5* gene as plant reporter systems inside the T-DNA borders. Mid-log phase bacteria (OD600 ≈ 1.0) cultured on Yeast Extract Broth (YEB) (14.0 gL^{-1} Nutrient broth, 1.0 gL^{-1} yeast extract, 10 mM $MgSO_4$ and 5.0 gL^{-1} sucrose, pH7.5) were used for all transformation experiments. Samples were cultured at 26 ºC under 16 hours light / 8 hours dark photoperiod with a light intensity of 31.4 µmol $m^{-2}s^{-1}$ in the growth room. For infection, one SCV of the suspension cells was submerged in four volumes bacterial broth after the liquid media was completely removed by careful pipetting. Bacteria broth was completely removed and co-cultivation media (MS liquid media) was added after infection. Co-cultivation was carried out at 28 ºC in darkness. For post co-cultivation treatments, cells were washed in liquid media supplemented with appropriate concentrations of hygromycin and 300 mgL^{-1} cefotaxime as selection media (SM) placed on a rotary shaker at 80 rpm

for one hour. Cells were then transferred into 50 ml of SM medium and maintained for 20 days prior to sub-culturing and plating. Cells were subsequently plated on solidified SM media (SMA) supplemented with 2% (w/v) Gelrite^{TM} (Duchefa, Netherlands) (named SMA, thereafter) for recovery and regeneration. Data was scored by counting the regenerants recovered on SMA selection plates. Transformation efficiency was expressed as number of regenerants per ml of SCV. Each treatment was done in triplicates and the experiment was repeated three times. Statistical analysis was done using ANOVA (SPSS, Inc, US) with Duncan's multiple comparison test at 95% confidence level.

3.3.3 GUS Histochemical assessment and GFP visualisation of putative transformed suspension cultures

For GUS histochemical staining, transformed suspension cultures were stained in histochemical reagent containing 0.1 M phosphate buffer, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.1 % (v/v) Triton X- 100, 10.0 mM EDTA, 20 % (v/v) methanol and 1.0 mM 5-Bromo-3-indolyl-glucuronide (X-Gluc) (Fermentas, US) (Appendix G). Explants were incubated at 37 ^{o}C in darkness for 4 – 24 hours until blue colouration appeared. Stained samples were then transferred and washed in 70 % (v/v) ethanol. Finally, stained samples were transferred and fixed in Formalin/ Acetic/ Alcohol (FAA) solution as described in Jefferson *et al*. (1987). The GUS stains were then examined under a contrast phase light microscope (Carl Zeiss model) and photographed. For GFP detection, cells were visualised under 460 nm excitation UV wavelength with an inverted microscope equipped with a cool camera.

3.3.4 Molecular assessment

3.3.4.1 Plasmid extraction

Plasmid DNA was prepared using the method of alkaline lysis as described by Sambrook *et al*. (1989). One ml of overnight cultured bacterial cells was aliquoted into sterile microcentrifuge tubes. The cells were then centrifuged at 10,621×*g* for 1 min in a microcentrifuge (Eppendorf 5430 R, Germany). Pellets of bacterial cells were then resuspended in 100 µl of pre-cold Solution I and incubated on ice for 5 mins after brief vortexing. After 5 min, 200 µl of freshly-prepared Solution II was added. The contents were then inverted gently 4 – 5 times followed by 5 min incubation on ice. An aliquot of 150 µl of pre-cold Solution III was then added and inverted for 5 times. The mixtures were then placed on ice for 10 min. The mixtures were then centrifuged at 17,949 ×*g* for 4 mins. Approximately 400 µl of the supernatant were retrieved and transferred to a new microcentrifuge tube. An equal volume of phenol was added in fume cupboard and mixed well. The mixtures were then centrifuged at 17,949 ×g for 2 min. The aqueous phase (upper part) was then transferred to a new tube and an equal volume of chloroform was added. The mixtures were then centrifuged at 17,949 ×*g* for 2 min after mixed well. Two volumes of cold absolute ethanol (EtOH) were added to the supernatant and transferred into new tubes. The mixtures were then left on ice for 10 mins and later centrifuged at 17,949 ×*g*, 4 °C for 8 min. After that, 1000 µl of 70 % (v/v) EtOH was added after draining away all the absolute EtOH inside the microcentrifuge tube. The mixtures were then centrifuged at 17,949 ×*g*, 4 °C for 3 min after mixed well. Finally, the pellet was dried completely by inverting on the paper towel and 50 µl of Tris-EDTA (TE) buffer (pH 8.0) was added to resuspend it. One µl of 20 µg/ ml ribonuclease (RNase) was pipetted carefully into the final product and incubated at room temperature for 20 min. Gel electrophoresis analysis (as described in Section 3.5.2) was then carried out to check the integrity of the plasmid extracted.

3.3.4.2 Gel electrophoresis

Extracted plasmid DNA, plant DNA and PCR products were analysed and visualised with Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA) on different concentration of agarose gel electrophoresis as indicated in Table 3.1. Agarose gel was prepared by dissolving an appropriate amount of agarose powder in 40 ml of 0.5X Tris-Acetate EDTA (TAE) buffer (Sambrook *et al.*, 1982). DNA samples to be analysed were mixed with 6X loading dye (Promega, USA) at a ratio of 4 vol. DNA: 1 vol. of loading dye before being loaded into the wells. Agarose gel electrophoresis was then executed at 90 Volts for 35 min and then viewed, photographed and analysed using Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA).

Table 3.1: Concentrations of agarose gel used for different types of DNA samples

Samples	Percentage (w/v) of agarose used for gel electrophoresis (%)
Plasmid DNA	0.8 %
Plant DNA	0.7 %
PCR products	1.0 %

3.3.4.3 Plant DNA extraction and quantification

Plant DNA was extracted with a modified Doyle and Doyle method (1987). Approximately 2 g of leaf materials were ground in the presence of liquid nitrogen (N_2) using mortar and pestle. Ground powder of plant materials were then transferred into Falcon tubes containing 10 ml of CTAB homogenization buffer (Appendix E). Homogenates were then incubated in a water bath at 65 ºC for 60 mins. Ten ml of Chloroform: Isoamyalcohol (24: 1) were then added and the mixtures were inverted gently for 10 mins. The mixtures were then centrifuged at 3,824 ×*g* for 15 mins. Supernatants were retrieved and transferred into new tubes. 2/3 volume of pre-cold Isopropanol was added into the supernatant and mixtures were kept at – 20 ºC overnight to precipitate DNA. Then, the supernatants were discarded after 15 mins of centrifugation at 3,824 ×*g*. Resulting pellet was then washed with 70 % (v/v) EtOH and transferred into a new 1.5 microcentrifuge tube. Washed pellet was dried after centrifugation at 17,949 ×*g* for 10 min. Finally, 500 µl of TE buffer and 2 µl of RNase were added to the DNA samples. DNA samples were then incubated at room temperature for RNase to work before storing at – 20 ºC. Extracted DNA samples were quantified using a Biophotometer (Eppendorf, USA) at wavelengths of 260 nm (OD_{260}) and 280 nm (OD_{280}). Concentration of DNA samples were then calculated as:

DNA Concentration (µg/ µl) = (OD_{260} × Dilution Factor × 50 µg / ml)/ 1000. Quality of DNA samples were indicated by the ratio of OD_{260} to OD_{280} (OD_{260} / OD_{280}). Integrity of DNA samples were checked by gel electrophoresis as described previously.

3.3.4.4 PCR confirmation of transformed cells

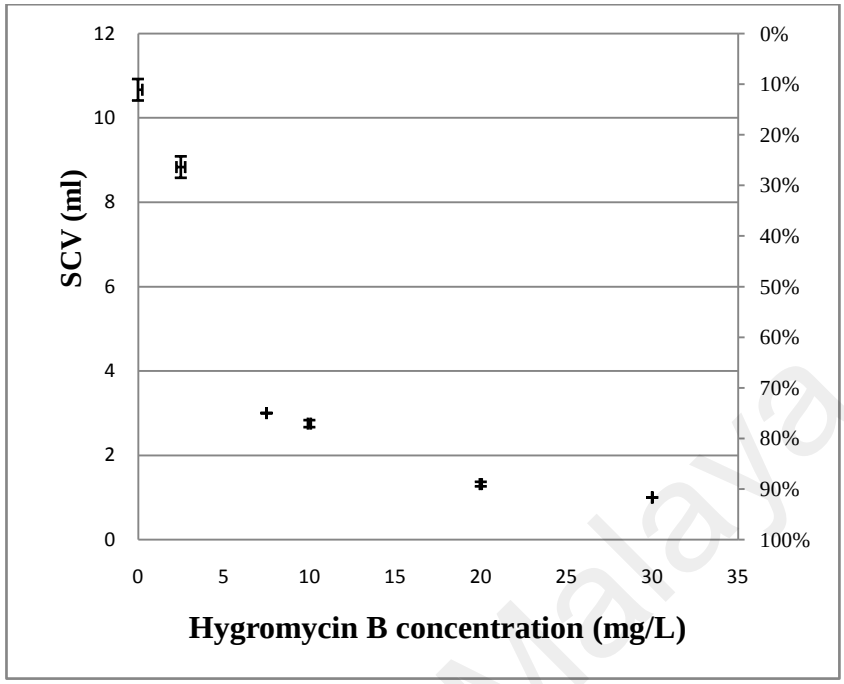
For PCR analysis, total genomic DNA was isolated according to modified Doyle and Doyle (1987) method. Equal amounts of 100 ng of total DNA were amplified in 20 µl reactions using a pair of primers specific to *mgfp5* gene: 5' – AAG GAG AAG AAC TTT TCA CTG GAG – 3' and 5' – AGT TCA TCC ATG CCA TGT GTA – 3' which were expected to give products of 700 bp. The PCR amplification was performed with an initial denaturation at 95 ^{o}C for 1 min, followed by 30 cycles at 95^{o}C for 1 min, 55 ^{o}C for 1 min, 68 ^{o}C for 1 min, and a final extension of 68 ^{o}C for 10 min. PCR products were then separated on 1.0% agarose gel.

3.4 Results and Discussion

3.4.1 Minimal inhibitory concentration (MIC) of hygromycin B (HYG) against *B. rotunda* cells

This experiment was carried to ensure successful selection of transformed cells and to eliminate non-transgenic cells from further analysis. Optimum concentration of the antibiotics was determined as too high concentration can be toxic and causes abnormalities in plants, but chances of false positives are high if otherwise. The effectiveness of the selection agents was assessed by determining the MIC and natural tolerance of the cell cultures (Parveez *et al*., 2007).

Growth of *B. rotunda* cells was inhibited when HYG was applied in SM media for about 20 days (Fig. 3.4a). Effective inhibition was found at 20 mgL^{-1} which corresponded to a lethal dose higher than 75% (LD_{75}). While, the effects of HYG in SMA as shown in Fig. 3.4b. LD_{75} of the cells was found to be at 30 mgL^{-1} HYG when the cells were plated. Higher concentration of HYG was required in SMA than in SM. Figure 3.5 showed that the cells survived and recovered on the selection media plates supplemented with 0 mgL^{-1} (Control), 10 mgL^{-1}, 20 mgL^{-1}, 30 mgL^{-1} and 50 mgL^{-1} HYG. Necrotic cells eventually turned brown and died (shown in red arrows). No surviving cells were found on SMA supplemented with 50 mgL^{-1} HYG. Gradual increase in HYG concentration for effective selection was also observed in carnation (Kinouchi *et al*., 2006). In this study, the application of HYG was adjusted for selection at different developmental growth phases i.e. propagation and regeneration of the suspension cell in liquid media SM and solid agar plate, respectively. The concentration of 20 mgL^{-1} and 30 mgL^{-1} were finally applied for effective selection of transgenic *B. rotunda* cell cultures.



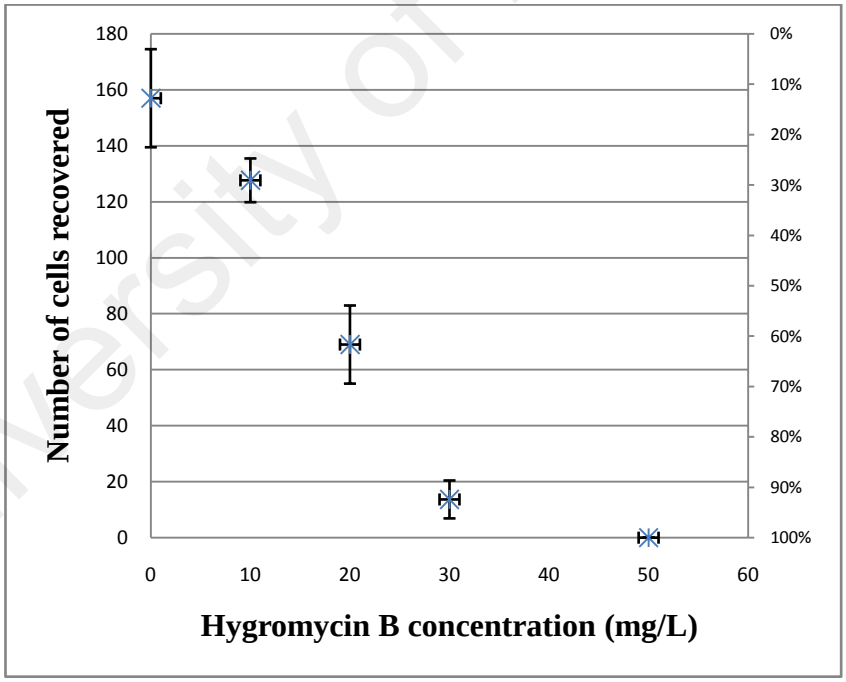


Figure 3.4: Inhibitory effects of hygromycin B against *B. rotunda* suspension culture in (a) liquid media SM and (b) solid agar plate SMA. LD_{75} = Lethal Dose 75%.

3.4.2 *Agrobacterium*-mediated transformation efficiency of *B. rotunda* suspension cell

In *Agrobacterium*-mediated transformation, infection time and co-cultivation period are important parameters affecting transformation efficiency (Gelvin, 2003). The effects of infection times and co-cultivation periods on the cell are shown in Fig. 3.6. The highest number of cells recovered on selecton plates after transformation was achieved with 10 mins of infection time and 2 days of co-cultivation with *Agrobacterium*. The transformed cells recovered after subsequent selection on SM and SMA are shown in Fig. 3.7a & 3.7b. Embryoid structures appeared after 3 – 4 weeks of plating while the non-transformed cells turned brown or pale and eventually died. Cells subjected to GFP visualisation are shown in Fig. 3.7c. Transformed cells appeared fluorescent green under UV excitation. The tissues subjected to GUS histochemical staining are shown in Figs. 3.7d. The gene (*gus*) transformed into the cells encoded the enzyme β-glucuronidase which catalyses the substrate X-Gluc giving rise to insoluble blue precipitates in the cell (Jefferson, 1989). Selected regenerated cells were then subjected to PCR analysis. The bands corresponding to ≈700 bp PCR products were observed (Fig. 3.8), confirming the presence of the transgene (*mgfp5*) in the cells.

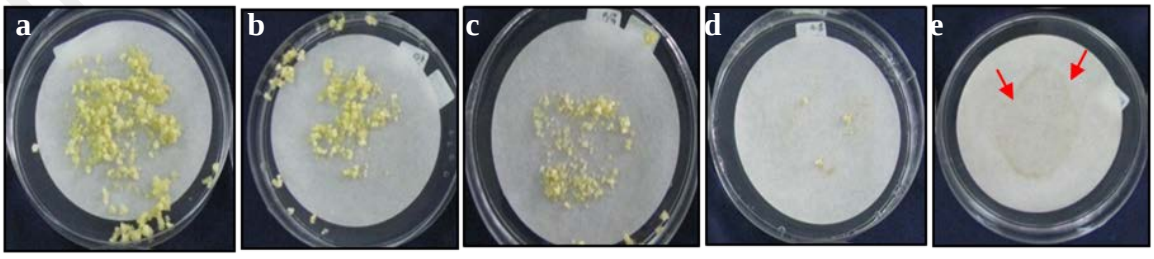


Figure 3.5: Cells subjected to HYG selection in SMA supplemented with different concentrations of hygromycin B. (a) 0 mg L^{-1} (Control), (b) 10 mg L^{-1}, (c) 20 mg L^{-1}, (d) 30 mg L^{-1} and (e) 50 mg L^{-1}. Red arrows indicate browning cells which eventually retarded to grow.

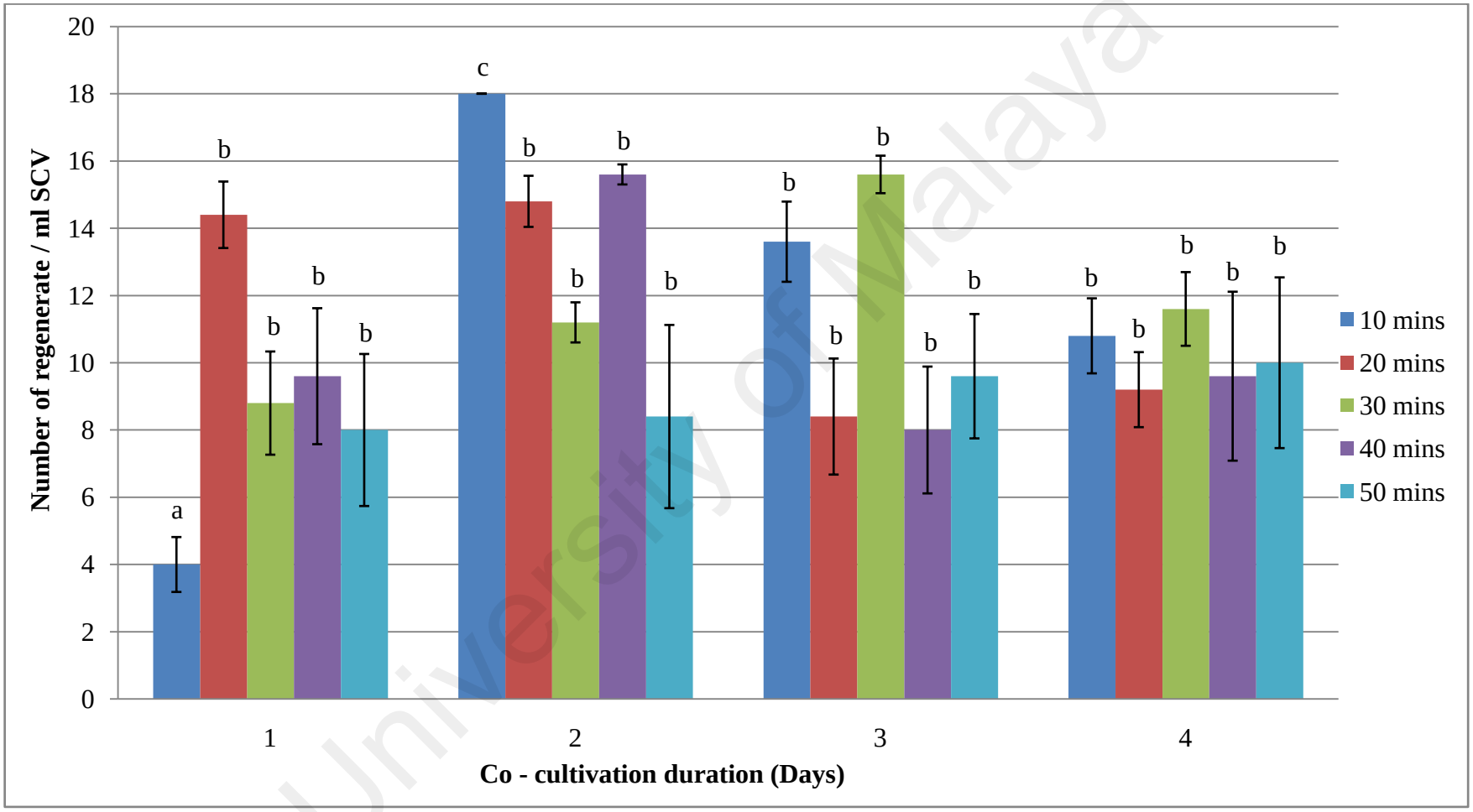


Figure 3.6: The effects of infection times and co-cultivation period on *Agrobacterium*-mediated transformation of *B. rotunda* suspension cell.

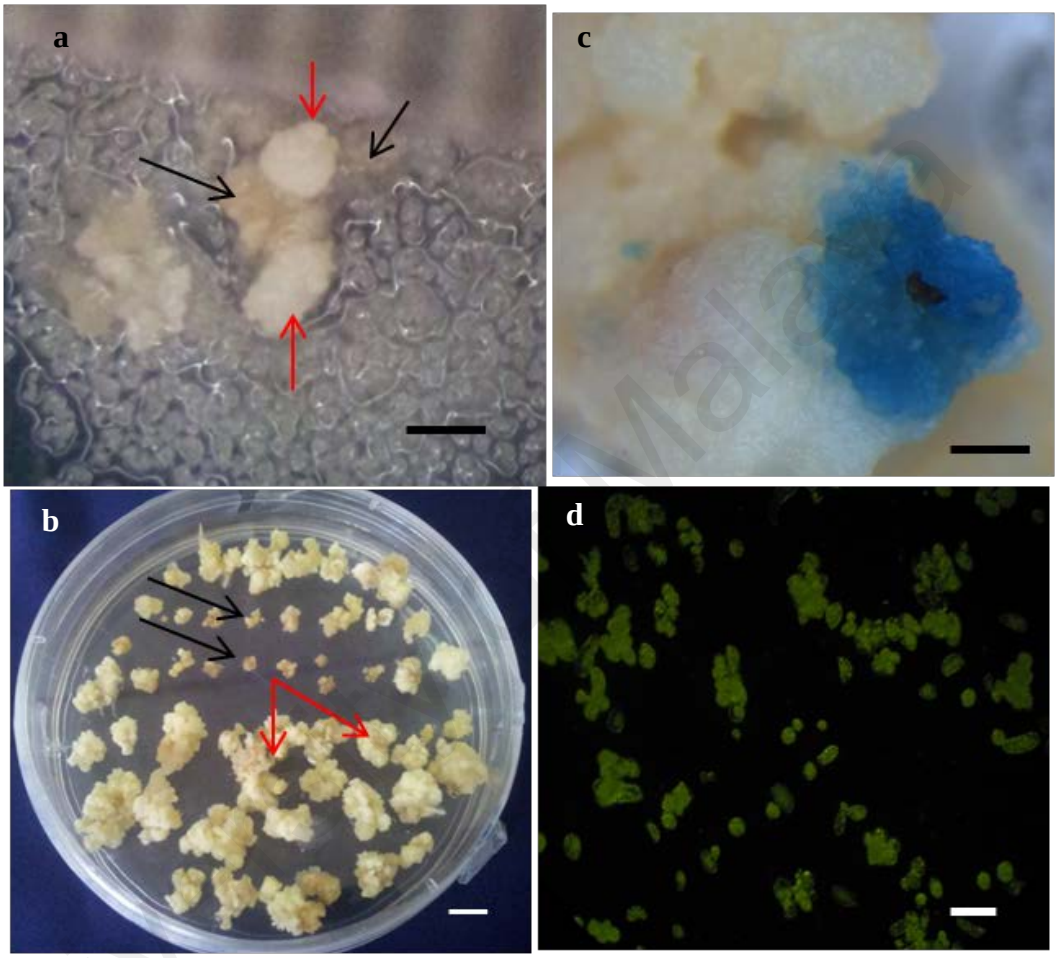


Figure 3.7: Hygromycin selection, GUS histochemical and green fluorescent assays: (a) & (b) Embryoid regenerants (red arrows) and non-transformed cells (black arrows) on hygromycin selection, bar = 1 mm, 1 cm. (c) Transformed cells stained blue in GUS histochemical assay, bar = 1 mm. (d) Transformed cells appear green fluorescent under UV excitation, bar = 10 µm.

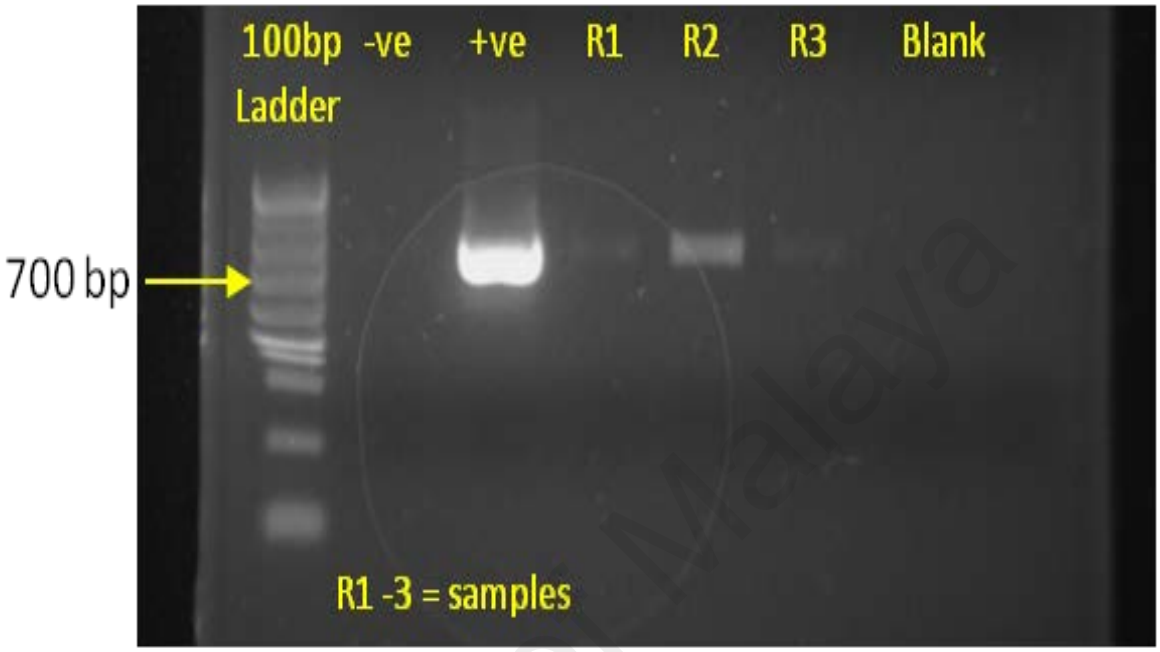


Figure 3.8: PCR analysis of transgenic *B. rotunda* cell suspension cultures. Lane 1 = 100 bp DNA Ladder, 2 = negative control (wild-type cells), 3 = plasmid as positive control, 4, 5, 6 = Samples, 7 = Blank.

CHAPTER 4: MOLECULAR CLONING AND RNAI KNOCKDOWN OF C4H (CINNAMATE-4-HYDROXYLASE) IN *B. ROTUNDA* CELL SUSPENSION CULTURES

4.1 Introduction

4.1.1 RNA silencing in plants

The phenotype of RNA silencing was first described as a mystery by Wingard (1928) in a tobacco plant infected with tobacco ringspot virus that acquired immunity and resistance to secondary infection on upper leaves of the plant. The solution to the mystery eventually became apparent from extensive studies carried out on viral defence and its mechanisms in plants (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Jones and Dangl, 2006; Waterhouse and Helliwell, 2001). It is well known that RNA silencing protects plants against viruses, protects the genome from transposable elements and most importantly regulates gene expression (Alba *et al.*, 2013).

Plants are special as compared to other organisms which lost one or more of the silencing pathways, they retained the three types of natural silencing pathways: 1- cytoplasmic siRNA silencing, 2- endogenous miRNA-mediated silencing and 3- DNA methylation and transcription suppression silencing (Baulcombe, 2014). In mammals, for example, almost all the natural silencing studied involved endogenous miRNAs (Bartel, 2004).

The silencing pathways share some similar key components, such as Dicer (RNase-III like dsRNA-specific ribonuclease) and AGO protein member from the Argonaute gene family. The biogenesis of RNA silencing involving siRNAs can be illustrated in the model proposed in figure 4.1 (Hutvágner and Zamore, 2002), this type of silencing is often referred as RNA interference (RNAi) (Wang *et al*., 2000).

Related machinery and mechanism involved in the pathway are summarised in Table 4.1. When homologous mRNA degradation is triggered by dsRNA (double-stranded RNA) or hpRNA (hairpin RNA), the mechanism can be divided into two steps: initiation and effector steps (Cerutti, 2003). In the initiation step, the inducers, short interfering RNA or microRNA (siRNA or miRNA) are produced by an Dicer-like complex and incorporated as a guide into an RNA-induced silencing complex (RISC) to knockdown or knockout homologous mRNA expression (Lu *et al.*, 2004). In the effector step (also called amplification step), RNA-dependent RNA polymerases (RdRPs) mediate primer-dependent or –independent amplification of silencing (Dalmay *et al.*, 2000). Secondary inducers produced by RdRPs extend the silencing effects which also can be spread systemically (Molnar *et al.*, 2011).

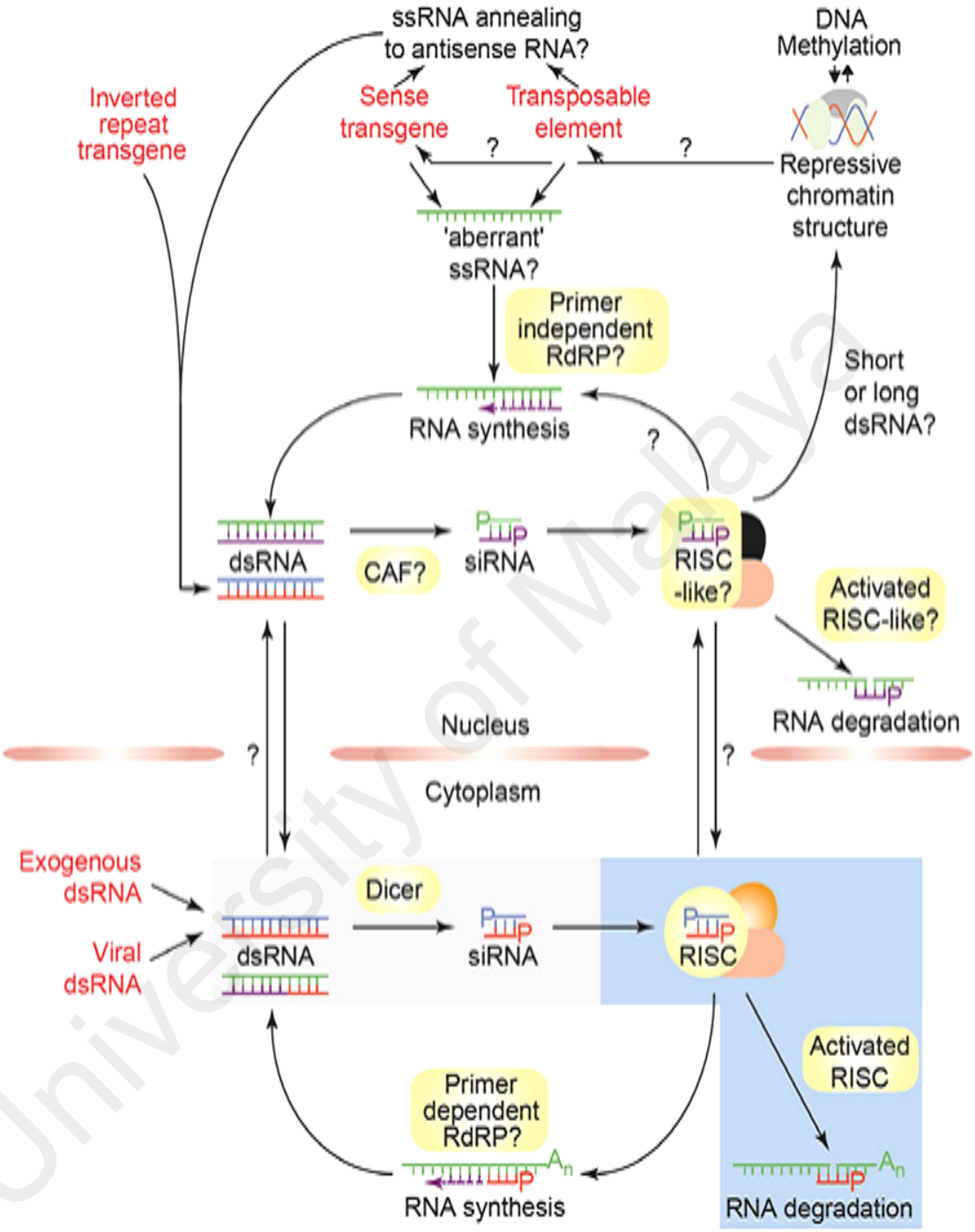


Figure 4.1: A model of siRNA molecular pathways proposed in Hutvágner and Zamore (2002).

Table 4.1: Summary of plant RNA silencing pathways machinery and mechanism

RNA silencing pathways	Inducer involved	Signature targets or effects	References
Cytoplasmic siRNA silencing	siRNA (21-26 nt)	Formation of aberrant RNA species, secondary structures of the RNA e.g. dsRNA or hpRNA which processed into siRNA	Broderson and Voinnet, 2006; Hamilton *et al.*, 2002; Lindbo and Dougherty, 1992; Napoli *et al.*, 1990
MiRNA-mediated silencing	miRNA (21-24 nt)	Negatively regulate gene expression by base pariring with complementary ss mRNA	Kim, 2005; Lippman and Martienssen, 2004; Reinhart *et al*., 2002; Zilberman *et al.*, 2003
DNA methylation/ transcription suppression	siRNA (24 nt) or miRNA	Trigger siRNA-directed DNA methylation or heterochromatization, can leads to genome rearrangements	Li et al., 2005; Onodera *et al.*, 2005; Wassenegger *et al.*, 1994; Yu *et al*., 2005

4.1.2 Applications of RNA silencing/ RNAi technology and metabolic engineering in plants

From the advancement of RNA silencing studies, plant biologists have continued to exploit this powerful tool for crop improvements and gene functional studies (Small, 2007). One of the successful applications of gene silencing in plants is protection of plants from viruses. Potatoes resistant to potato leafroll virus and papaya resistant to papaya ringspot virus are among the earliest outcome of silencing-conferred virus resistance in transgenic plants (Fuchs and Gonsalves, 2007). The transgenic papaya has been proven a great success in rescuing papaya industry, even though the mechanism is not well-characterized yet (Fuchs and Gonsalves, 2007). Wang *et al*. (2000) performed the first deliberate use of RNA silencing to produce barley yellow dwarf virus –resistant barley using a hpRNA-encoding construct targeting the 5' end of the virus. Besides, RNAi application had also extended to protect plants from organisms other than viruses, such as parasitic nematodes (Fairbairn *et al*., 2007; Hoffman *et al*., 2008), corn rootworm (Baum *et al.*, 2007) and cotton bollworm (Jin *et al*., 2015).

Crop improvement through RNAi has not only limited to plant protection from invasion, but also improves nutritional value and modification of metabolic pathway. The application has benefited food crop and non-food crop species as well as medicinal plant species to produce valuable metabolite compounds. Table 4.2 shows the examples of crop improvement and metabolic engineering efforts through RNAi by fine tuning of the enzyme(s) involved in the biosynthesis pathways.

RNAi endeavours have also facilitated the revealing of gene functions in plant functional genomics studies (Sato, 2005). Comprehensive analysis of Quantitative Trait Loci (QTL) or multigene family effects could be evaluated via RNAi because of the sequence specificity of silencing in QTL loci or multigene families (Kusaba, 2004).

RNAi is advantageous for crop improvement over conventional mutational breeding and knockout mutants which are usually associated with unexpected outcomes (Ifuku *et al.*, 2003; Miki *et al.*, 2005). Unexpected outcomes are more often undesirable, for example lethality, extreme pleiotropic phenotypes and abnormal feedback mechanisms which could be avoided because RNAi silencing effect is sequence-specific (Small, 2008; Voinnet *et al.*, 1998). Therefore, it may be a method of choice to complement the existing knockout technology for genes that are not amenable to knockout technology.

Several approaches have been developed for efficient RNAi in plants i.e. hpRNA vector via *Agrobacterium*-mediated transformation, virus-induced gene silencing (VIGS) via virus vectors, and direct synthetic dsRNA or the vector containing the dsRNA sequences or siRNA induced gene silencing method via particle bombardment (Sato, 2005). Stable transformation could be achieved via *Agrobacterium* (Allen *et al.*, 2008; Nakatsuka *et al.*, 2010) while VIGS (Baulcombe, 1999; Burch-Smith *et al.*, 2004; Scofield *et al.*, 2005) and direct introduction via particle bombardment (Voinnet *et al.*, 1998; Schweizer *et al.*, 2000; Shim *et al.*, 2012) are usually transient (Watson *et al.*, 2005).

Transient RNAi has been useful for gene functions screening (An *et al.*, 2005; Dubouzet *et al.*, 2005), however, for most metabolic engineering investigation, stable transformation via *Agrobacterium* is more desirable (Allen *et al,*. 2008; Diretto *et al.*, 2007; Kempe *et al.*, 2009; Larkin *et al.*, 2007; Nakatsuka *et al.*, 2010; Park *et al.*, 2002, 2003). Nevertheless high throughput gene function screening could be carried out via transient RNAi for identification of metabolic target while compounds production could be executed via a stable metabolic engineering method.

Table 4.2: Examples of crop improvement efforts through RNAi of the enzyme involved in the biosynthesis pathways

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Catharanthus roseus *	Tryptophan decarboxylase	Tryptamine	Production of nonnatural alkaloids in plant culture	Runguphan *et al.* (2009)
Eschscholzia californica *	Berberine bridge enzyme (BBE)	Benzophenanthridine alkaloids	Reduced accumulation of benzophenanthridine alkaloids but reduced cell growth rate	Park *et al*. (2002); Park *et al*. (2003)
Eschscholzia californica *	BBE	(S)-reticuline	Accumulation of reticuline which is a key compound for isoquinone alkaloids synthesis	Fujii *et al*. (2007)
Fragaria x ananassa	Anthocyanin reductase (ANR)	Anthocyanins	Premature, ectopic anthocyanin formation and shortened chain lengths of proanthocyanidins observed	Fisher *et al*. (2014)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Gentiana triflora x Gentiana scabra cv Albireo	Anthocyanin 5,3'-aromatic acyltransferase and flavonoid 3',5'-hydroxylase	Anthocyanin	Flower colour changed (pale-blue and lilac instead of blue)	Nakatsuka *et al.* (2010)
Glycine max	Isoflavone synthase	Isoflavones	Major inhibition of isoflavone accumulation and renders the plant susceptible to *Phytophythora sojae*	Subramanian *et al.* (2005)
Glycine max L. Merril	β-amyrin synthase	Saponin	Reduction of seed saponin content with no abnormality found in seed development and growth	Takagi *et al.* (2011)
Gossypium hirsutum cv Coker 315	Fatty acid desaturase genes (Δ9-desaturase and ω6-desaturase)	Palmitic acid	Increased stearic acid content 2-40% and oleic acid up to 77%	Liu *et al.* (2002)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Jatropha curcas	Sugar-dependent 1	Triacylglycerol lipase	Enhanced seed oil accumulation in seeds	Kim *et al.* (2014)
Juglans regia	Polyphenol oxidase (PPO)	PPO-derived quinones	Caused spontaneous necrotic lesions on leaves, major alterations in phenolic compounds metabolism and phenylpropanoid pathway genes expression	Araji *et al.* (2014)
Linum usitatissimum	Cinnamoyl alcohol dehydrogenase	Lignin reduction	Reduction in the lignin level associated with increase in the lignin precursor contents and a reduction in the pectin and hemicellulose constituents	Wrobel-Kwiatkowska *et al.* (2007)
Linum usitatissimum L.	Beta subunit of farnesyltransferase	Lignan	Increased content of lignan in transgenic calli	Corbin *et al.* (2013)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Linum usitatissimum L.	Pinoresinol lariresinol reductase	(+)-Secoisolariciresinol diglucoside (SDG), Lignans and neolignans	Increased accumulation of the pinoresinol substrate under its diglucosylated form and caused new compounds synthesis	Renouard *et al.* (2014)
*Mentha x piperita**	Cytochrome P450 (+) menthofuran synthase	Menthofuran	Reduction on undesirable monoterpenes	Mahmoud and Croteau (2001)
*Mentha x piperita**	Limonene-3-hydroxylase	Limonene	Accumulation of limonene up to 80% compared to about 2% in wild type plants without influence on oil yield	Mahmoud *et al.* (2004)
Nicotiana benthamiana	Aspartate aminotransferases	Essential amino acids within plastid	Disruption of phenylalanine metabolism and lignin deposition	Torre *et al.* (2014)
*Nicotiana tabacum**	Putrescine N-methyltransferase	Pyridine and tropane alkaloids	Elevated levels of anatabine	Chintapakorn and Hamill (2003)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Oncidium hybrid orchid	Phytoene synthase (PSY)	Carotenoids	Reduced total carotenoids contents, declined of the photosynthetic electron transport efficiency and developed semi-dwarf and brilliant green leaves	Liu *et al.* (2014)
Ophiorrhiza pumila	Tryptophan decarboxylase (TDC), secologanin synthase (SLS)	Camptothecin	Reduced accumulation of camptothecin and related alkaloids, strictosidine, strictosamide, pumiloside, and deoxypumiloside	Asano *et al.* (2013)
Panax ginseng *	Dammarenediol synthase	Ginsenoside	Reduction of ginsenoside production to 84.5% in roots and thus confirmed the role and importance of the enzyme in ginsenoside biosynthesis	Han *et al.* (2006)

Table 4.2 Continued

*Papaver somniferum**	BBE	Alkaloid	Increased several pathway intermediates. Altered morphinan and tetrahydrobenzylisoquinoline alkaloid in latex but not benzophenanthridine alkaloids in roots	Frick *et al*. (2004)
*Papaver somniferum**	Codeinone reductase	Codeine, morphine	Accumulation of the precursor alkaloid (S)-reticuline and nonnarcotic alkaloid reticuline	Allen *et al*. (2004)
*Papaver somniferum**	Codeinone reductase	Morphinan alkaloid	Accumulated 15% and 30% higher content of morphinan alkaloid	Larkin *et al.* (2007)
*Papaver somniferum**	Salutaridinol-7-O-acetyltransferase	Morphinan alkaloids	Significant accumulation of the alkaloid salutaridine up to 23% of total alkaloid	Allen *et al*. (2008)
*Papaver somniferum**	Salutaridinol-7-O-acetyltransferase	Morphine	Led to accumulation of intermediate compounds	Kempe *et al.* (2009)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Petunia x hybrida	Benzoic acid/ salicyclic carboxyl methyltransferase	Methylbenzoate	Reduction of methylbenzoate emission 75% to 99% and implicated	Underwood *et al*. (2005)
Petunia hybrida	Benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase	Benzylaldehyde	Reduction in benzylbenzoate formation and increased benzyl alcohol and benzylaldehyde concentration	Orlova *et al*. (2006)
Petunia hybrida	Coniferyl alcohol acyltransferase	Coniferyl aldehyde and homovanilic acid	Inhibition of isoeugenol biosynthesis and suggested that coniferyl acetate is the substrate of isoeugenol synthase	Dexter *et al.* (2007)
Petunia hybrida	R2R3 MYB-type transcription factor (*ODORANT1*)	Fragrance	Reduced volatile benzenoid levels through decreased synthesis of precursors and suggested that the gene is a key regulator for fragrance biosynthesis	Verdonk *et al.* (2005)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Petunia hybrida	Phenylacetaldehyde synthase	Phenylacetaldehyde and 2-phenylethanol	Complete suppression of the products	Kaminaga *et al.* (2006)
Rosa hybrid	Dihydroflavonol 4-reductase (DFR)	Delphinidin	Combine with over-expression of the enzyme DFR from iris and viola flavonoid 3'5'-hydroxylase produced pure blue rose flower	Katsumoto *et al.* (2007)
Salvia miltiorrhiza	Chalcone synthase (CHS)	Phenolic acids and flavonoids	Enhanced phenolic acids contents and decreased the accumulation of total flavonoids	Zhang *et al.* (2015)
Solanum lycopersicum	Cinnamoyl-CoA reductase	Phenolic compounds	Elevation of total soluble phenolics compounds and triggered accumulation of two metabolites in vegetative organs	Van der Rest *et al.* (2006)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Solanum lycopersicum	De-etiolated 1	Carotenoid and flavonoids	Increased both carotenoid and flavonoid contents in fruits using fruit-specific promoters combined with RNAi vector	Davuluri *et al.* (2005)
Solanum lycopersicum	Chalcone synthase	Flavonoids	Pathernocarpic (seedless) tomato fruits	Schijlen *et al.* (2007)
Solanum lycopersicum	Lycopene beta-cyclase	Lycopene	Slight increase in lycopene content	Rosati *et al.* (2000)
Solanum lycopersicum	12-oxophytodienoic acid reductase3 (OPRs)	Jasmonic acid (JA)	OPR3-RNAi plants contained wild-type levels of OPDA but failed to accumulate JA after wounding, reduced trichome formation and impaired monoterpene and sesquiterpene production	Bosch *et al.* (2014)
Solanum tuberosum	β-carotene hydroxylase, non-heme iron hydroxylases and cytochrome P450	Carotenoids and β-carotene	Enhancement of tuber carotenoid content	Diretto *et al.* (2007)

Table 4.2 Continued

Plant species	Enzyme(s)/ gene(s) involved	Target products	Significant outputs	References
Solanum tuberosum	Hydroxycinnamoyl CoA: quinate hydroxycinnamoyl transferase (HQT)	Chlorogenic acid (CGA)	Reduction of CGA and early flowering	Payyavula *et al.* (2015)
Torenia fournieri	Glucose 6-phosphate/ phosphate translocator gene	Anthocyanin	Inhibition of anthocyanin systhesis and chlorophyll degradation	Nagira *et al.* (2006)
Tritucum aestivum	Starch-branching enzyme II a and b	Starch composition	High-amylose wheat line	Regina *et al*. (2006)

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4.1.3 RNAi vectors and the pANDA vector

Binary vector systems are the most established method for *Agrobacterium*-mediated transformation since 1990s. However, this method is time-consuming and laborious for high-throughput cloning of target sequences. RNAi vector construction is also restricted when limited by available restriction enzyme sites and problems due to in large plasmid size (Karimi *et al.*, 2002). Therefore, researchers have developed Gateway cloning technology to ease high-throughput generation of inverted-repeat vectors by site-specific recombination (Hartley *et al.*, 2000). These vectors have served many purposes including RNAi, overexpression (Xiao *et al.*, 2012), protein localization, promoter functional analysis, artificial miRNA-mediated gene silencing study (Carbonell *et al.*, 2014), as well as protein/protein interactions analysis (Gehl *et al.*, 2011).

All Gateway-compatible vectors are derivatives of the backbone pPZP200 vector which has been widely used for high-throughput cloning of target sequences (Birch, 1997). The gateway vectors are the 'destination' vector, which receives the target sequences cloned in an 'entry' vector. The gateway vector "pANDA" developed by Miki and Shimamoto (2004) was chosen to be used in this study, the map and features of this plasmid ere shown in figure 4.2. This plasmid is about 20 kbp, contains Kanamycin and Hygromycin resistance genes for bacteria and plant selection, has a maize ubiquitin promoter, a NOS terminator, a partial GUS linker gene in between the clonase recombinase sites for generation of hpRNA of the target sequences, and left border (LB), right border (RB) for plant genome integration. This vector was used successfully for *Agrobacterium* RNAi transformation in rice (Ishimaru *et al.*, 2013; Miki and Shimamoto, 2004; Miki *et al.*, 2005; Satoh-Nagasawa *et al.*, 2013), Switchgrass (Fu *et al.*, 2011), wheat (Cruz *et al.*, 2014), barley (*H. vulgare* L. cv. Morex) (Zheng *et al.*, 2011) and Arabidopsis (Xing *et al.*, 2013).

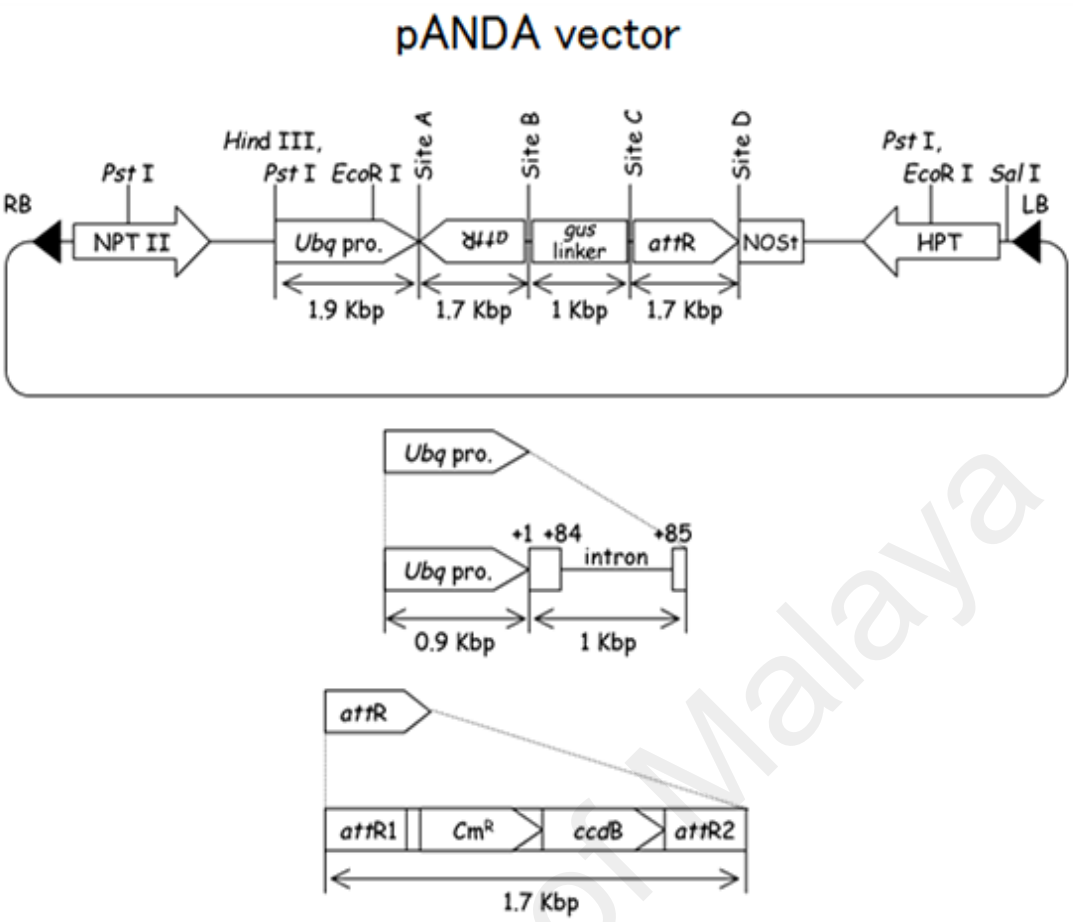


Figure 4.2: pANDA vector map

LB: left border

RB: right border

NPT II: Kanamycin resistance gene

HPT: Hygromycin resistance gene

Ubq pro.: Maize ubiquitin1 promoter + 1st intron & splicing acceptor site

attR: LR clonase recombination cassette

attR1 & attR2: LR clonase recombination sites

CmR: Chloramphenicol resistance gene

ccdB: ccd B gene

NOSt: NOS terminator

Vector size: about 20 Kbp

Back bone: pBI101

Host *E. coli* strain: DB 3.1

Unique restriction enzyme sites: Kpn I and Sac I

(Miki and Shimamoto, 2004)

4.1.4 The enzyme cinnamate 4-hydroxylase (C4H)

Phenylpropanoid compounds are derived from phenylalanine, the precursor of phenylpropanoid pathway (KEGG: EC00940). Phenylalanine is converted into cinnamic acid by the action of phenylalanine ammonia-lyase (PAL), which is then hydrolysed into ρ-coumaric acid via the enzyme C4H and then activated by the enzyme 4-coumarate coenzyme A (CoA)-ligase (4CL) to give rise to its thioester before being channelled into different branches in the pathway (Fig. 4.3). The reaction of C4H activity is shown in figure 4.4.

The enzyme C4H (EC: 1.14.13.11) is involved in the second step in the pathway, and is a cytochrome P450 shown to be highly responsive to light, wounding and infection (Chapple, 1998) and thus plays an important role in plant defence from UV and pathogens (Ahuja *et al.*, 2012). C4H cDNAs have been isolated from several plant species, including Arabidopsis (Bell-Lelong *et al.*, 1997; Raes *et al.*, 2003), Populus (Lu *et al.*, 2006), *Brassica napus* (Chen *et al.*, 2007), rice (Yang *et al.*, 2005), red sage (Huang *et al.*, 2008), apricot and plum (Pina *et al*., 2012).

Most of the C4Hs are extremely conserved at both the protein and nucleotide levels, containing conserved hinge motif at the N terminus and a heme-binding domain required for catalytic activity at the C terminus (Chen *et al.*, 2007; Pina *et al.*, 2012). Phylogenetic analysis revealed that the dicot and monocot C4H enzymes have no clear difference (Xu *et al.*, 2009) but can be categorised in two classes based on functionality (Nedelkina *et al.*, 1999; Pina *et al.*, 2012). Class I C4Hs are typical secondary metabolism–related cinnamate hydroxylases while Class II C4Hs are cell differentiation and developmental stages–related (Nedelkina *et al.*, 1999; Betz *et al.*, 2001).

C4H enzyme is also involved in pathways other than phenylalanine metabolism and phenyplpropanoid biosynthesis, for instance ubiquinone and terpenoid-quinone

biosynthesis (KEGG, AT2G30490; Kanehisa Laboratories, 2015) as well as stilbenoid, diarylheptanoid and gingerol biosynthesis (KEGG, AT2G30490; Kanehisa Laboratories, 2013).

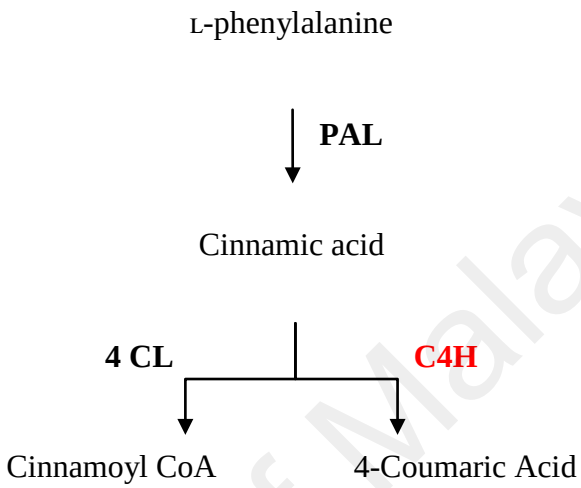
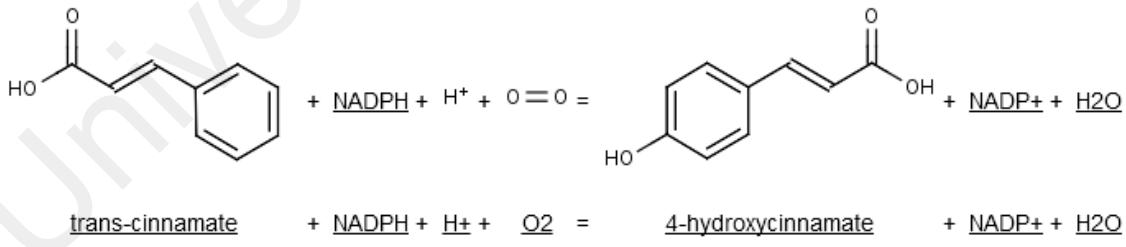


Figure 4.3: C4H enzyme as a central branch in the phenylpropanoid pathway.



Trans-cinnamate + NADPH + H^+ + O_2 ←→ 4 –hydroxycinnamate + $NADP^+$ + H_2O

Figure 4.4: Reaction catalysed by C4H

(Brenda, 2016)

4.2 Objectives of the study

1. To clone C4H cDNA and subclone into a RNAi construct for generating inverted repeat of partial C4H gene
2. To introduce the construct into *Agrobacterium* LBA4404
3. To knock-down or knock-out C4H gene expression in *B. rotunda* cell suspension cultures
4. To evaluate the C4H-dsRNA RNAi effects on gene expression in relation to phenylpropanoid biosynthesis and related compound production

4.3 Materials and Methods

4.3.1 Cloning and isolation of C4H gene

4.3.1.1 RNA preparation and gene cloning

Standard gene cloning methods (Sambrook *et al*., 1989) were used to prepare the gene constructs. Cell suspension culture was used as the source of plant material for cloning and isolation of C4H cDNA. Plant RNA was extracted using Qiagen plant RNeasy mini kit according to manufacture manual. DNase I treatment was carried out to eliminate traces of DNA by adding 1 µl each of 10X DNase I reaction buffer and DNase I to 1 µg of total RNA extracts in appropriate amount of double-autoclaved nuclease-free water (nf-H_2O) and incubated for 15 min at room temperature. Reaction was stopped by adding in 1 µl of 25 mM EDTA and followed by 10 min incubation at 65°C for 10 mins in a dry bath. The quality of the extracts was assessed by Experion^{TM} gel analyzer (Bio-Rad Laboratories, Inc, US) and Nanodrop 2000 UV-Vis Spectrophotometer (Fisher Science Education, US). RNA extracts of good purity ($OD_{260/280} \approx 2.0$) and integrity (RQI > 8) were chosen for further experiments in Real-time qPCR analysis. At the meantime, the RNA extracts were stored in -80^{o}C. For cDNA conversion, a total amount of 100 ng RNA was adjusted in 5 µl nf-H_2O and added into 10 µl of High Capacity RT master mix containing buffer, dNTP mix, random primers, and reverse transcriptase. Reverse transcription was carried out in a thermalcycler with the following incubations: 25^{o}C for 10 min, 37 ^{o}C for 120 min, 85 ^{o}C for 5 min and hold at 4 ^{o}C. The cDNA were then kept in a freezer until further use.

4.3.1.2 Primer design and gene cloning

Primer pairs were designed with the Primer3 software (Koressaar and Remm, 2007; Rozen, 2012) from transcriptome data provided in the laboratory (Md. Mustafa *et al.*, 2014). The resulting primer pair sequences are shown in Table. 4.5. Seven sets of primers were used in the isolation of partial C4H gene. PCR and RT-PCR (Reverse transcription – PCR) was carried out in 20 µl reactions using a thermalcycler (Eppendorf®, US). The amplification was performed with an initial denaturation at 95 ^{o}C for 1 min, followed by 30 cycles at 95^{o}C for 1 min, 55 ^{o}C for 1 min, 68 ^{o}C for 1 min, and a final extension of 68 ^{o}C for 10 min. PCR products were then separated on 1.5% agarose gel and purified using INtron purification kit according to the method described by the manufacturer and sent for DNA sequencing at Bioneer Corporation, Republic of Korea. Sequence processing and analysis were carried out using BioEdit Sequence Alignment Editor to obtain longer fragment of C4H cDNA and the sequences were subjected to blastx and blastn in NCBI and Phyre software for identification and confirmation.

4.3.1.3 Full length gene cloning using Rapid Amplification of cDNA Ends (RACE) method

Rapid Amplification of cDNA Ends (RACE) is a technique used to obtain full length sequence of an RNA transcript based on RNA-ligase-mediated and oligo-capping rapid amplification of cDNA ends methods. The RNA sequence of interest is produced from a pool of cDNA produced via reverse transcription, followed by PCR amplification of the 5’ end or 3’ end of the cDNA. The amplified cDNA copies are then sequenced and mapped to mRNA sequence available from the database or other resources. Expressed sequence tags (EST), subtracted cDNA, differential display or library screening can be the source of RNA pool which provide information of differential transcription and regulation. Besides, this method can be used to identify the 5’ and 3’ untranslated

regions of genes, to study heterogenous transcriptional start sites and to characterise promoter regions. This method was used in the study to obtain the cDNA sequence of the enzyme cinnamate-4-hydroxylase (C4H) for designing a Taqman probe for C4H gene expression quantification in transformed ginger suspension cell. For 5'-RACE, RNA was treated with calf intestinal phosphate (CIP) to dephophorylate non-mRNA or truncated RNA and subsequently, mRNA cap structure were removed using Tobacco Acid Pyrophosphatase (TAP). 5' GeneRacer^{TM} (Invitrogen, US) RNA oligo was then ligated to decapped RNA using 5 Unit of T4 RNA ligase in 10 µl reaction mix containing ligase buffer, ATP and 40 Unit of RNaseOUT^{TM}. Reaction was carried out at 37°C for 1 hour. RNA was then purified by phenol:chloroform method according to manufacture manual. Finally, RNA pellet was re-suspended in 10 µl of nf-H2O and analysed 1 µl by agarose gel electrophoresis. For cDNA conversion, 10 µl RNA ligated with GeneRacer^{TM} RNA oligo was added to 1 µl of random primers, 1 µl of dNTP mix and 1 µl of nf-H2O. The mixture was incubated at 37°C for 5 min to remove any RNA secondary structures and chilled on ice for about 1 min. High Capacity RT master mix containing buffer and reverse transcriptase, DTT and RNaseOUT^{TM} were then added to the mixture and the mixture was briefly centrifuged to bring all ingredients to the bottom of the tube. Reverse transcription was carried out in a thermalcycler with following incubation: 25^{o}C for 5 min, 50 ^{o}C for 60 min, 70 ^{o}C for 15 min and hold at 4 ^{o}C. One µl of RNase H (2U) was added into the reaction mix and incubated at 37 ^{o}C for 20 min. For 3'-RACE, dephophorylation and decapping of the RNA were unnecessary while the RNA was reverse transcribed using GeneRacer^{TM} oligo dT to replace GeneRacer^{TM} RNA oligo with the same conditions as above. To amplify cDNA ends, RACE-ready cDNA with known priming sites on each end was used to amplify the 5' end and 3' end using GoTaq Flexi (Promega, US) according to the following PCR reaction mix in Table 4.3 and PCR condition in Table 4.4. The control HeLa total RNA

provided in the kit was included in all RACE-PCR reaction using GeneRacer^{TM} 5' primer and GeneRacer^{TM} 3' primer to amplify the HeLa β-actin cDNA. The amplified products were analysed with agarose gel electrophoresis. Products with distinct band were purified directly while products with multiple bands were cut from the gel before further purification using the reagents included in the kit. Purified products were sent sequencing and the sequencing results were analysed using Blastn and Blastx provided in NCBI and ClustalW 2.1 (Larkin *et al*., 2007). Nucleotide to protein sequence translation was done using ExPaSy translate tool (ExPASy; Gasteiger *et al*., 2003) and protein sequences were analysed using protein folding and homology recognization tool, Phyre2 (Kelley *et al*., 2015).

Table 4.3: PCR reaction mix

Reagents	**5' RACE**	**3'RACE**
GeneRacer™ 5'primer, 10 µM	3 µl	-
C4H reverse primer (GSP), 10 µM	1 µl	-
GeneRacer™ 3'primer, 10 µM	-	3 µl
C4H forward primer (GSP), 10 µM	-	1 µl
cDNA template	1 µl	1 µl
5X Flexi buffer	10 µl	10 µl
$MgCl_2$,	5 µl	5 µl
dNTP mix	1 µl	1 µl
GoTaq polymerase (5U/ µl)	0.5 µl	0.5 µl
Sterile nf-H_2O	28.5 µl	28.5 µl
Total	**50 µl**	**50 µl**

Table 4.4: PCR condition

Standard condition			**Manufacture recommendation**		
Temperature	**Time**	**Cycles**	**Temperature**	**Time**	**Cycles**
94 ^{o}C	1 min	1	94 ^{o}C	2 min	1
94 ^{o}C	1 min	30	94 ^{o}C	30s	5
55 ^{o}C	30 s		72 ^{o}C	1 min	
68 ^{o}C	1 min		94 ^{o}C	30s	5
72 ^{o}C	10 min	1	70 ^{o}C	1 min	
0 ^{o}C	Hold		94 ^{o}C	30s	20-25
			60 ^{o}C	30s	
			72 ^{o}C	1 min	
			72 ^{o}C	10 min	1
			0 ^{o}C	Hold	

4.3.2 Generation of the C4H-hpRNA RNAi vector and transformation of *Agrobacterium*

The gene sequence for which inverted repeats are made was then amplified by a proofreading DNA polymerase (PFU Taq, Promega®, US). The "CACC" sequence is added to the 5' end of forward primer which is required for providing the right direction of the PCR product in the TOPO® vector. The DNA fragment was gel-purified using Wizard PCR Preps DNA Purification System (Promega). For subcloning of the PCR product in the entry clone, PCR product was mixed with a directional TOPO pENTER vector (pENTR/D-TOPO), and transformed into TOP10® *E. coli* competent cells (Invitrogen, US), according to the manufacturer's manual. Transformants are selected with 50 μg/ml kanamycin desired colonies were tested for the presence of partial C4H fragment using PCR amplification with the gene specific primers mentioned. The pENTR directional TOPO vector with C4H fragment was then mixed with RNAi vector of choice, pANDA vector plasmid in the presence of Gateway LR clonase enzyme mix (Invitrogen), which promotes in vitro recombination between entry clone and destination clone resulting in a hairpin construct, named as pANDA-C4H hereafter. The pANDA vector was a gift from the Laboratory of Plant Molecular Genetics, Nara Institute of Science and Technology, Ikoma, Japan, a derivative from Gateway ® vectors from Invitrogen. Reaction mixtures were prepared and according to the manufacturer's protocol. The mixture was transferred to competent *E. coli* DH5α strain (Promega) by heat-shock transformation, according to the manufacturer's protocol. Plasmid was then extracted and further confirmed by PCR using specific primers for the presence of the C4H fragment. Afterwards, *Agrobacterium tumefaciens* LBA4404 was transformed by heat-shock method (Hofgen and Willmitzer, 1988). The *A. tumefaciens* harbouring pANDA-C4H vector was then selected using 50 mgL^{-1} kanamycin, 50 mgL^{-1} hygromycin and 100 mgL^{-1} streptomycin. PCR analysis was then carried out to confirm

the presence of the plasmid in *A. tumefaciens* LBA4404 transformed into *A. tumefaciens* LBA4404 carrying the empty pANDA vector without C4H fragment also prepared and confirmed with PCR analysis. Benedict's test was performed for confirming the *Agrobacteria* according to the method described by Bernaerts and De Ley (1963).

4.3.3 Introducing the RNAi vector into *B. rotunda* suspension cell via *Agrobacterium*-mediated transformation

Agrobacterium culture preparation and suspension cell transformation were carried out according to the protocol optimised in Chapter 3 (Section 3.3). Cells were plated onto selection medium (SM media) containing 25 mgL^{-1} hygromycin B and 300 mgL^{-1} cefotaxime for selection. Callus grew on the selection media were transferred onto fresh SM media every two weeks. To obtain one cell line, single colony callus was selected under the microscope, labeled and propagated in selection media.

4.3.4 Molecular analysis

4.3.4.1 PCR analysis

Total genomic DNA was isolated with Doyle and Doyle (1987) method. DNA quality and quantity were accessed using a NanoDrop 2000 UV-Vis Spectrophotometer (Fisher Science Education, US). Equal amounts of 100 ng total DNA were amplified in 20 µl reaction using GoTaq® DNA polymerase mix (Promega, USA) and a pair of primers specific to the gus linker (*gusl*) sequence: 5'-CAT GAA GAT GCG GAC TTA CG-3' and 5'-ATC CAC GCC GTA TTC GG-3' which give expected products of ≈ 630 bp. The PCR amplification was performed with an initial denaturation at 95 ^{o}C for 1 min, followed by 30 cycles at 95^{o}C for 1 min, 60 ^{o}C for 1 min, 72 ^{o}C for 1 min, and a final extension of 72 ^{o}C for 10 min. PCR products were then separated in 1.0% agarose gel and visualised with Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA). C4H

gene primers (C4H 1F and 1R, Table 4.5) were also used in the PCR for each sample in parallel as an endogenous control.

Table 4.5: Primers set sequences used in cloning and isolation of *B. rotunda* C4H gene

Primers Name	Sequence (5' – 3')	Tm (°C)	CG%	Product Length (bp)
1F	GTC AAG TTC GGA CAG TTA CC	45.9	50.0	708
1R	CTC CAA GTA GCC TTT CAA GA	46.4	45.0	
2F	TGT CCT TCA ATC TCT ACT CCT CC	51.0	47.8	917
2R	TCC TCC ATC ATC TTC CTG TTC G	55.5	50.0	
4F	ATG ATG GAG GAA ATG GGA TC	50.0	45.0	406
4R	CGT TGA CCA GGA TCT TGC T	52.9	52.6	
5F	TCA TGA TCC GCC TCA TCT T	50.0	47.4	435
5R	ATC TGA TCC AGT ACC ATC GTC G	53.0	50.0	
6F	GTA GCG TTG CTT TTC ACA AT	47.4	40.0	598
6R	GAG AAC AAG ATC CTG GTC AA	46.2	45.0	
7F	TCG TTT CCA CCC TGT CCT T	51.7	52.6	1469
7R	CGC TTG TAC TTT CCG TAT CTG	49.6	47.6	
9F	CGC CGG AAT CTT TAC AAT TAC C	54.4	45.5	1351
9R	CCA CAA CGT CGT CTC TAT CG	50.5	55.5	

4.3.4.2 Southern Blotting

Genomic DNA of transformed *B. rotunda* suspension cells were digested with the restriction enzymes *SacI* and *kpnI* (Fisher Scientific) according to the manufacturer's descriptions. Ten μg genomic DNA was used for each sample for standardisation. Digested DNA was size-separated using gel electrophoresis at 90 V for 30 mins. Separated fragments of DNA in 1% (w/v) agarose gel were then depurinated in 0.2 M HCl solution for 30 min, denatured into single-stranded (ss) DNA in denaturation buffer (1.5 M NaCl and 0.5 N NaOH) for 30 min (2 times) and then neutralized in 1 M Tris pH7.4 and 1.5 M NaCl with agitation for 30 min. Separated ssDNA were then transferred onto nitrocellulose membrane (Hybond-N, Amersham, US) using a capillary transfer method for 16 to 48 hrs. Paper cuts were changed from time to time to provide better transfer capillary action from the agarose gel to the membrane. Membrane was briefly air-dried and UV cross-linking was carried out at 1.5J/ cm^3 for 3 min. Probes were biotin-labelled by random priming with exonuclease activity-free Klenow fragment using primers specific to *gusl* gene sequence according to manufacturer's description (PureExtreme^{TM}, Fermentas, US). Hybridisation was then carried out by labelled-probes in hybridisation buffer (6X SSC, 1% (w/v) SDS and 0.01M EDTA) at 42 °C in a hybridisation oven with agitation for 16 hrs. Membrane was washed twice with 2X SSC, 0.1% (w/v) SDS at room temperature for 10 mins followed by 20 min high stringency wash with 0.1X SSC, 0.1% (w/v) SDS at 65 °C for 20 min. Chromogenic detection was carried out using Streptavidin-conjugated Alkaline Phosphatase which cleaves BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) to give an insoluble blue precipitate (Biotin Chromogenic Detection Kit, Thermo Fisher Scientific, US). Membrane was incubated with BCIP-T substrates solution at room temperature in the dark until blue coloration formation. Finally, the reaction was

stopped by removing the substrate solution and the membrane was rinsed several times with dH_2O and blotted dry on tissue paper.

4.3.4.3 Quantitative RT-PCR (qPCR) analysis of C4H expression

RNA was prepared as described in 4.3.1.1. Only RNA samples of good purity (OD260/280 ≈2.0) and integrity (RQI > 8) were used in qRT-PCR analysis. For cDNA conversion, a total amount of 100 ng RNA was adjusted in 5 µl nf-H_2O and added into 10 µl of High Capacity RT master mix containing buffer, dNTP mix, random primers, and reverse transcriptase. Reverse transcription was carried out in a thermalcycler with following incubation: 25^{o}C for 10 min, 37 ^{o}C for 120 min, 85 ^{o}C for 5 min and hold at 4 ^{o}C. Resulting cDNA were then kept in freezer until further use. Quantitative RT-PCR was carried using predesigned Taqman probes for C4H gene expression assay kit and normalized with endogenous β-actin gene expression (Applied Biosystems, Life Technologies, US). Each sample was tested in quadruplicate and reaction mixtures was prepared according to the kit descriptions. Samples were aliquoted into low profile capped tube strips and loaded into an ABI 7500 real-time PCR machine. The reaction was set as: hold at 50^{o}C for 2 min, 95 ^{o}C for 20 s, and run at 95 ^{o}C for 1 s and 60 ^{o}C for 20 s for 40 cycles. Relative quantification of the gene expression level was calculated using ABI 7500 System Sequence Detection software v1.2 (ABI, US)

4.3.4.4 Northern blotting

Northern blotting was carried out to detect the presence of the siRNA generated from gene silencing C4H dsRNA in *B. rotunda* cell suspensions transformed with *Agrobacterium* carrying the knock down construct, pANDA-C4H. Denaturing PACE gel was prepared using 15% polyacrylamide, 8M Urea in 0.5X TBE and polymerization was initiated by adding 0.1% Ammonium Persulfate and 10 µl of TEMED (tetramethylethylenediamine). RNA samples were heated at 65 ^{o}C for 10 mins in a heat

block to remove any secondary structure, mixed with equal volume of 2X loading dye (2X TBE, 40% sucrose, 0.1% bromophenol-blue) and size-fractionated using the denaturing PAGE gel at 200 V for 1.5 hr. For transferring of the RNA onto nitrocellulose membrane, a Bio-Rad Trans-Blot Semi-Dry Transfer Unit (Bio-Rad, US) was used. Membrane and 3 mm Whatman chromatography filter papers were pre-soaked in 0.5X TBE transferring buffer and sandwiched the gel on top of the platform of the semi-dry apparatus without formation of any bubbles between the filter paper-gel-membrane-filter paper layer. TBE buffer (0.5 X) was added to moist the sandwich layer. The transfer was carried out at constant current for 35 min and the voltage was remained as 20V. Biotin-labelled probes were prepared by random priming with exonuclease activity-free Klenow fragment using primers specific to C4H1 (Table 4.5, 1F and 1R) according to manufacturer's description (PureExtremeTM, Fermentas, US). After the transfer was complete, the gel sandwich was disassembled and the gel was soaked in ethidium bromide and check for complete transfer. The membrane was briefly air-dried and UV cross-linked at 1.5 J/ cm^3 for 3 min. Prehybridisation was carried out using hybridisation buffer (6X SSC, 1% (w/v) SDS and 0.01M EDTA) and 1 mg of sonicated Herring sperm DNA (Promega, Thrermo Fisher Scientific, US) was used for blocking purpose at this stage. Hybridization was then carried out in hybridisation buffer containing labelled-probes at 50 °C in hybridisation oven with agitation for overnight. After hybridisation, membrane was washed twice with 2X SSC, 0.1% (w/v) SDS at room temperature for 10 mins followed by 20 min high stringency wash with 0.1X SSC, 0.1% (w/v) SDS at 65 °C for 20 min. Chromogenic detection was done using Streptavidin-conjugated Alkaline Phosphatase which cleaves BCIP-T to give an insoluble blue precipitate. Biotin-labelled probes with affinity to streptavidin bind to complementary ssDNA on the membrane and react to give a defined band at the site. Membrane was incubated with BCIP-T substrates solution at room temperature in the

dark until blue coloration forms. Finally, reaction was stopped by removing the substrate solution and membrane was rinsed several times with dH_2O and blotted dry on tissue paper.

4.3.5 Liquid Chromatography-Mass Spectrometry (LC-MS)

4.3.5.1 Compounds extraction

For compounds extraction, plant tissue samples were air-dried in an oven at 38 ^oC until constant weight. 200 mg of ground oven dried tissues was used for extraction process. Samples were extracted using 2 ml of 80% (v/v) methanol with 0.1% (w/v) butylated hydroxy toulene (BHT). Ribitol (0.2 mg ml^{-1}) and Biochanin A (0.02 ppm) were added as internal standards. The mixture was vortexed for 30 s and agitated in an incubator shaker at 500 rpm for 5 min. Subsequently, the mixture was sonicated for 5mins at 37 kHz, 10^oC. The mixture was centrifuged at 2,000 ×*g*, 4^oC for 10 min. Supernatant was collected and transferred into a new clean tube. The process of extraction was repeated two times and methanol was added each round of extraction with a ratio of 80% (v/v) (Neoh *et al*., 2013). All the supernatant were combined and dried using an oxygen free nitrogen blower (OFN) before LC-MS analysis.

4.3.5.2 LC-MS analysis of primary metabolites

Extracts were dissolved in 100 µl 50% ACN:H_2O prior to instrumental analysis. Samples were analyzed using Waters Acquity LC system coupled with Mass Spectra detector, Xevo TQs (Triple Quadrupole). Separation was carried our using Waters Acquity UPLC HSS T3 column (1.8 µm, 2.1 mm × 100 mm) with corresponding solvent A (0.1% formic acid (FA) in water) and solvent B (0.1% FA in acetonitrile). The solvent gradient starts with 95% A and changes to 60% A for 3min. At the 3rdmin, the solvent gradient was 5% A for 2 min before reverting to 95% A at the 5th min and hold for 7 min. The flow rate was set at 0.3mL/min with injection volume of 3 µl. Both

positive and negative ESI modes were used in the mass detector with desolvation temperature of 350^{o}C and the capillary voltage at 2.9 KV. All metabolites were optimized to obtained specific cone voltage and collision energy with automatic dwell time calculated. The total acquisition time was 15 min.

4.3.5.3 LC-MS analysis of secondary metabolites

For LC-MS analysis of secondary metabolites, the extracts were dissolved in 100 µl 50% ACN:H_2O prior instrumental analysis. Samples were analyzed using Waters Acquity LC-MS system coupled with Mass Spectra detector as above. Separation was carried out using Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm) with corresponding solvent A (0.1% formic acid (FA) in water) and solvent B (0.1% FA in acetonitrile). The solvent gradient starts with 60% A for 3 mins and decreased to 50% A for 7 min. At the 10th min, the solvent gradient was 30% A for 3 min before changing to 15% A and finally to 100% B on the 18th min. The flow rate was set at 0.3 ml min^{-1} with injection volume of 3 µl. Positive ESI (Electron Spray Ionisation) mode was used in the mass detector with desolvation temperature of 350^{o}C. Meanwhile the capillary voltage was set at 3.5 kV with cone voltage of 20 V. All metabolites were optimised to obtain specific cone voltage and collision energy with automatic dwell time calculated. The total acquisition time was 25 min.

4.3.5.4 LC-MS analysis of phenolic compounds

Extracts were dissolved in 100 µl 50% MeOH:H_2O prior to instrumental analysis. Samples were analysed using Waters Acquity LC-MS system as above. Separation was carried out using Waters UPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm) with corresponding solvent A (0.1% formic acid (FA) in water) and solvent B (0.1% FA in methanol (MeOH)). The solvent gradient starts with 88.5% A and changes to 50% A at the 4^{th} min. After 8 min, gradient A was reverted back to 88.5% A and hold for 3 min. The flow rate was set at 0.3 ml min^{-1} with injection volume of 3 µl. Negative ESI mode was used in the mass detector with desolvation temperature of 300^oC and capillary voltage at 3.0 kV. Both cinnamic and coumaric acid were optimized to obtained specific cone voltage and collision energy with automatic dwell time calculated. The total acquisition time was 15 min.

4.3.5.5 Statistical analysis on LC-MS data

Relative abundance (R/A) for each metabolite was obtained from the peaks area detected and compared with reference standard in the LC-MS system. The fold-change in metabolites was calculated as their log_2 ratio compared to the wild type sample and their *P*-value was analysed using T-test based on two-tailed distribution at 95% confidence level where n = 9 with 3 biological replicates.

4.4 Results and Discussion

4.4.1 C4H gene isolation and sequence analysis

Annealing temperature gradient PCR was carried out for each primer set in order to obtain the best condition for isolating C4H cDNA from *B. rotunda*. The result of the best annealing temperature for each set of primers and their respective PCR product lengths obtained from PCR is shown in figure 4.5 and summarised in Table 4.6. Fragments isolated using primer sets 1F & 1R, 7F & 7R and 9F & 9R were gel-purified and further subjected to RACE-PCR to obtain full length cDNA. DNA Sequencing revealed the sequences (Appendix B) and length of the RACE-PCR products (Table 4.7). The sequences were further analysed with Blastx, Blaxtp and Phyre2.

When the sequences were subjected to blastn similarity search, the three sequences amplified by primers sets 1, 7 and 9 showed high sequence similarity to known C4H in the NCBI Genebank (Tables 4.8, 4.9 and 4.10). The sequence C4H1 was determined to have 83% similarity with 92% coverage compared to *Musa acuminata* AAA Group cultivar Cavendish cinnamate 4-hydroxylase (C4H3) mRNA, partial cds [Genebank Accession No: KF582544.1], putative C4H protein in *Oryza sativa Japonica* [Genebank Accession No: NM 001053354.1], trans-cinnamate 4-monooxygenase-like mRNA in *Brachypodium distachyon* [Genebank Accession No: XM 003574905.1] and a putative cytochrome P450 in *Zea mays* [Genebank Accession No: NM 001147254.1].

The sequences C4H7 and C4H9 show sequence similarity to sequences coding C4H in many plant species such as *Eucalyptus urophylla*, *Musa acuminata*, *Brassica rapa* subsp. pekinesis, and *A. thaliana* at a coverage of <70%.

Sequence analysis of C4H1 using blastp revealed that the cloned sequence contains a putative conserved domain PLN02394, a trans-cinnamate 4-monooxygenase conserved domain which belongs to the cytochrome P450 superfamily (Fig. 4.6).

Phyre2 analysis showed that this sequence encoded for 316 amino acids which folded into protein primary structure and secondary structure based on a crystal structure of *Arabidopsis* cytochrome P450 which is a putative allene oxide synthase complexed in figure 4.7 (Appendix C). Thus, the results supported that the fragment cloned and isolated was a C4H enzyme and the gene fragment was used in RNAi experiments.

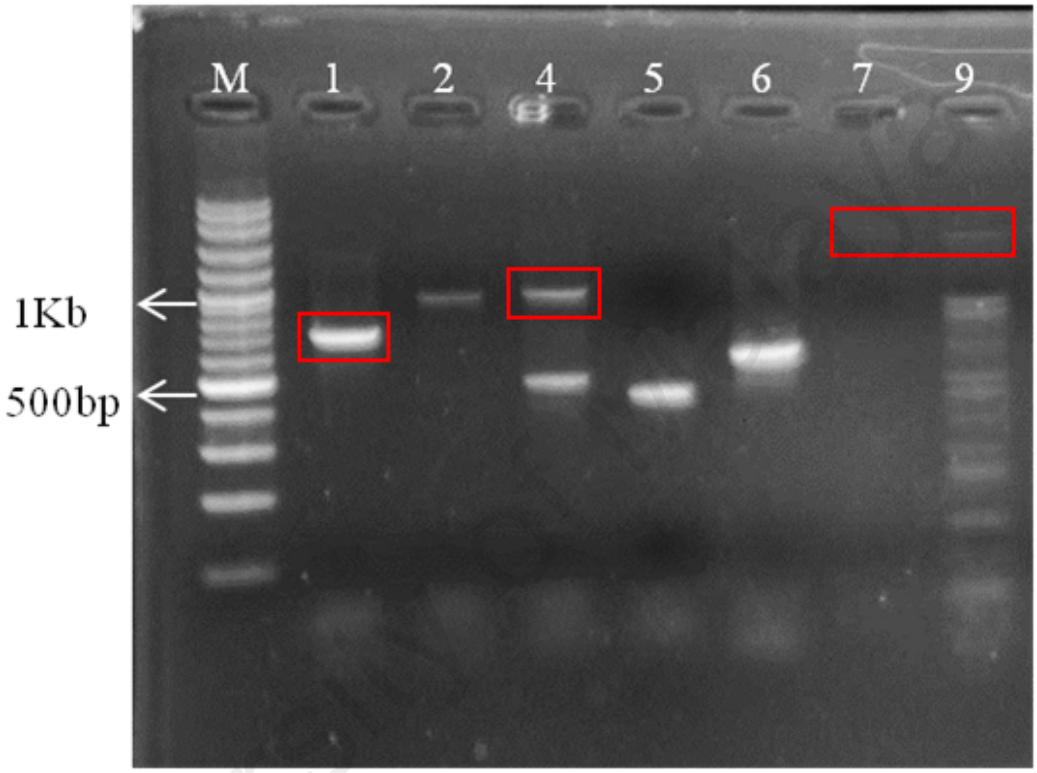


Figure 4.5: Gel electrophoresis of PCR products amplified using different primer pairs. Lane M is 100bp plus DNA marker. Lane 1 = Primers set C4H1, Lane 2 = C4H2, Lane 4 = C4H4, Lane 5 = C4H5, Lane 6 = C4H6, Lane 7 = C4H7 and Lane 9 = C4H9 were labelled according to the primers set numbered. DNA bands highlighted in red box were purified, amplified using RACE-PCR, sent for sequencing and subjected to further analysis.

Table 4.6: Seven primer pairs and their respective PCR products length

C4H Primer sets	T_m (°C)	Expected PCR Products Length (bp)	Products Length (bp)	RT-PCR Products Length (bp)
1F & 1R	56.5	708	~ 700	~ 700
2F & 2R	50.8	917	~ 1000	~ 1000
4 F & 4R	50.8-56.5	406	~ 1000 & 500	~ 1000 & 500
5 F & 5R	48.4-63.8	435	~ 450	~ 500
6 F & 6R	50.8-59.3	598	~ 600	~ 600
7 F & 7R	48.4-53.8 or 50.8	1469	~ 1500 and ~ 380	NIL
9 F & 9R	53.6-56.5	1351	1351	Multiple bands

Table 4.7: Summary of RACE-PCR results C4H clones

Primer sets	RACE-PCR Product Size (bp)	Clone Name
1 F & 1 R	1, 302	C4H1
7 F & 7 R	1, 300	C4H7
9 F & 9 R	1, 498	C4H9

Table 4.8: Sequence homology search (Blastn) results of C4H1

Description	Identity	Query Coverage	E value	Accession
Musa acuminata **AAA Group cultivar Cavendish cinnamate 4-hydroxylase (C4H3) mRNA, partial cds**	83%	92%	0.0	KF582544.1
PREDICTED: Musa acuminata **subsp. malaccensis cytochrome P450 CYP73A100-like (LOC103997903), mRNA**	83%	92%	0.0	XM 009419241.1
Oryza sativa **Japonica Group Os02g0467600 (Os02g0467600) mRNA, complete cds**	79%	87%	0.0	NM 001053354.1
Predicted: ***Brachypodium distachyon*** **trans-cinnamate 4-monooxygenase-like (LOC100832881), mRNA**	79%	81%	0.0	XM 003574905.1
Hordeum vulgare **subsp.** ***vulgare*** **mRNA for predicted protein, complete cds, clone: NIASHv2141C24**	78%	90%	0.0	AK371769.1
Hordeum vulgare **subsp.** ***vulgare*** **mRNA for predicted protein, complete cds, clone: NIASHv2047H04**	78%	90%	0.0	AK366849.1
Phyllostachys edulis **cinnamic acid 4-hydroxylase mRNA, partial cds**	76%	89%	3e-156	EU780142.1

Table 4.8 Continued

Description	Identity	Query Coverage	E value	Accession
Zea mays **trans-cinnamate 4-monooxygenase (LOC100282780), mRNA >qb[EU962294] Zea mays clone 241576 trans-cinnamate 4-hydroxylase**	76%	87%	1e-149	NM 001155686.1
Zea mays **putative cytochrome P450 superfamily (LOC100272801), >qb[BT039467] Zea mays full-length cDNA clone**	76%	87%	7e-148	NM 001147254.1
Predicted: ***Oryza brachyantha*** **trans-cinnamate 4-monooxygenase-like (LOC102768416), transcript variant X2**	76%	89%	9e-147	XM 006654198.1

91

Table 4.9: Sequence homology search (Blastn) results of C4H7

Description	Identity	Query Coverage	E value	Accession
Eucalyptus urophylla **cinnamate 4-hydroxylase (C4H1) gene, complete cds**	82%	39%	2e-107	JX270996.1
Aquilaria sinesis **cinnamate 4-hydroxylase (C4H) mRNA complete cds**	81%	38%	3e-102	KF134783.1
Musa acuminate **AAA Group cultivar Cavendish cinnamate 4-hydroxylase (C4H1) mRNA, partial cds**	87%	25%	2e-93	KF582542.1
Hordeum vulgare **subsp.** ***vulgare*** **mRNA for predicted protein, complete cds, clone: NIASHv2141K20**	79%	39%	7e-82	AK371802.1
Leucaena leucocephala **clone LIC4H1b cinnamate 4-hydroxylase (C4H) mRNA, complete cds**	80%	34%	3e-82	HQ191221.2
Leucaena leucocephala **cinnamate 4-hydroxylase (C4H1) mRNA, complete cds**	80%	34%	3e-77	GU183363.2
Hordeum vulgare **subsp.** ***vulgare*** **mRNA for predicted protein, complete cds, clone: NIASHv2141C24**	74%	36%	2e-34	AK366849.1
Hordeum vulgare **subsp.** ***vulgare*** **mRNA for predicted protein, complete cds, clone: NIASHv2047H04**	74%	36%	2e-34	AK366849.1
Brassica rapa **Br-cr-C4HL mRNA for cinnamate 4-hydroxylase, complete cds**	73%	34%	8e-33	AB427091.1

Table 4.9 Continued

Description	Identity	Query Coverage	E value	Accession
***Brassica rapa* subsp. pekinensis cinnamate 4-hydroxylase mRNA, complete cds**	73%	34%	4e-31	DQ457008.1
Predicted: *Brachypodium distachyon* trans-cinnamate 4-monooxygenase-like (LOC100832881), mRNA	73%	34%	4e-31	XM003574905.1

Table 4.10: Sequence homology search (Blastn) results of C4H9

Description	Identity	Query Coverage	E value	Accession
Musa acuminate **AAA Group cultivar Cavendish cinnamate 4-hydroxylase (C4H2) mRNA, partial cds**	83%	54%	6e-148	KF582543.1
Oryza sativa **Japonica Group genomic DNA, chromosom 1, BAC clone: OJ1529 G03**	77%	70%	1e-109	AP003446.3
Oryza sativa **Japonica Group Os01g0820000 (Os01g0820000) mRNA, complete cds**	80%	53%	6e-108	NM 001185699.1
Dendrobium officinale **cinnamate 4-hydroxylase mRNA, complete cds**	81%	46%	5e-104	KC713783.1
Aquilaria sinensis **cinnamate 4-hydroxylase (C4H) mRNA, complete cds**	79%	54%	2e-102	KF134783.1
Eucalyptus urophylla **cinnamate 4-hydroxylase 1 (C4H1), complete cds**	79%	54%	8e-102	JX270996.1
Leucaena leucocephala **clone LlC4H1b cinnamate 4-hydroxylase (C4H) mRNA, complete cds**	79%	35%	3e-61	HQ191221.2
Leucaena leucocephala **cinnamate 4-hydroxylase 1(C4H) mRNA, complete cds**	78%	35%	6e-58	GU183363.2
Festuca rubra **subsp. commutata CYP73A91-4 mRNA, complete cds**	85%	9%	9e-17	JF682489.1
Festuca rubra **subsp. commutata CYP73A91-3 mRNA, complete cds**	85%	9%	9e-17	JF682488.1

Table 4.10 Continued

Description	Identity	Query Coverage	E value	Accession
***Festuca rubra* subsp. commutata CYP73A91 mRNA, complete cds**	84%	9%	9e-17	JF682486.1
***Festuca rubra* subsp. commutata CYP73A91-2 mRNA, complete cds**	84%	9%	4e-15	JF682487.1
***Arabidopsis thaliana* chromosome 2, complete sequence**	83%	9%	2e-13	CP002685.1
***Arabidopsis thaliana* partial C4Hgene for cinnamate 4-hydroxylase, ecotype Gr-5, exons 1-3**	83%	9%	2e-13	AM887637.1
***Arabidopsis thaliana* C4Hgene for cinnamate 4-hydroxylase, ecotype Me-0, exons 1-3**	83%	9%	2e-13	AM887636.1

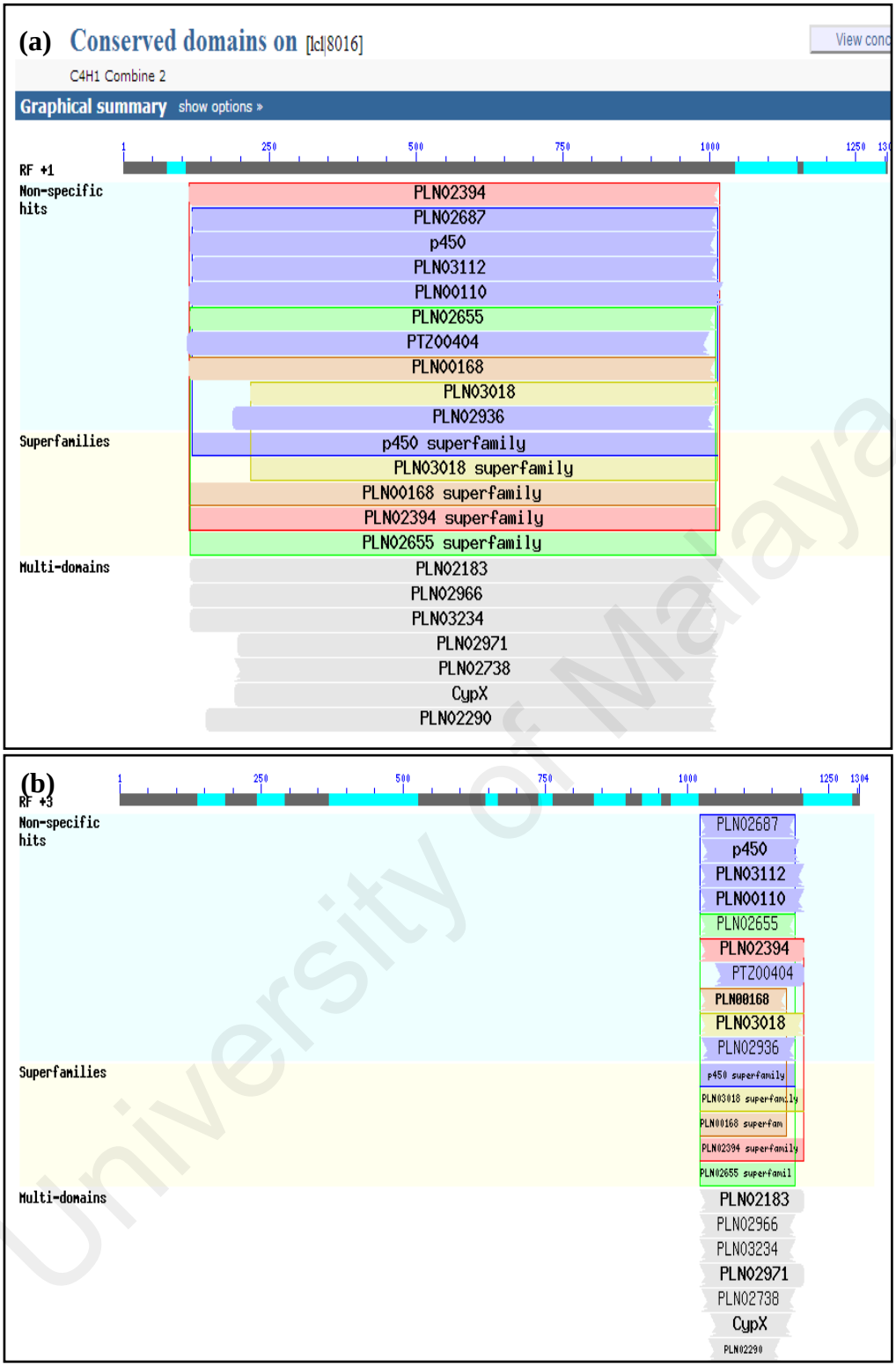


Figure 4.6: Putative domain search (blastp) of C4H1 partial cDNA sequence (a): Conserved domains for first 1000 bp of the sequence and (b): Conserved domains for downstream 300 bp of the sequence.

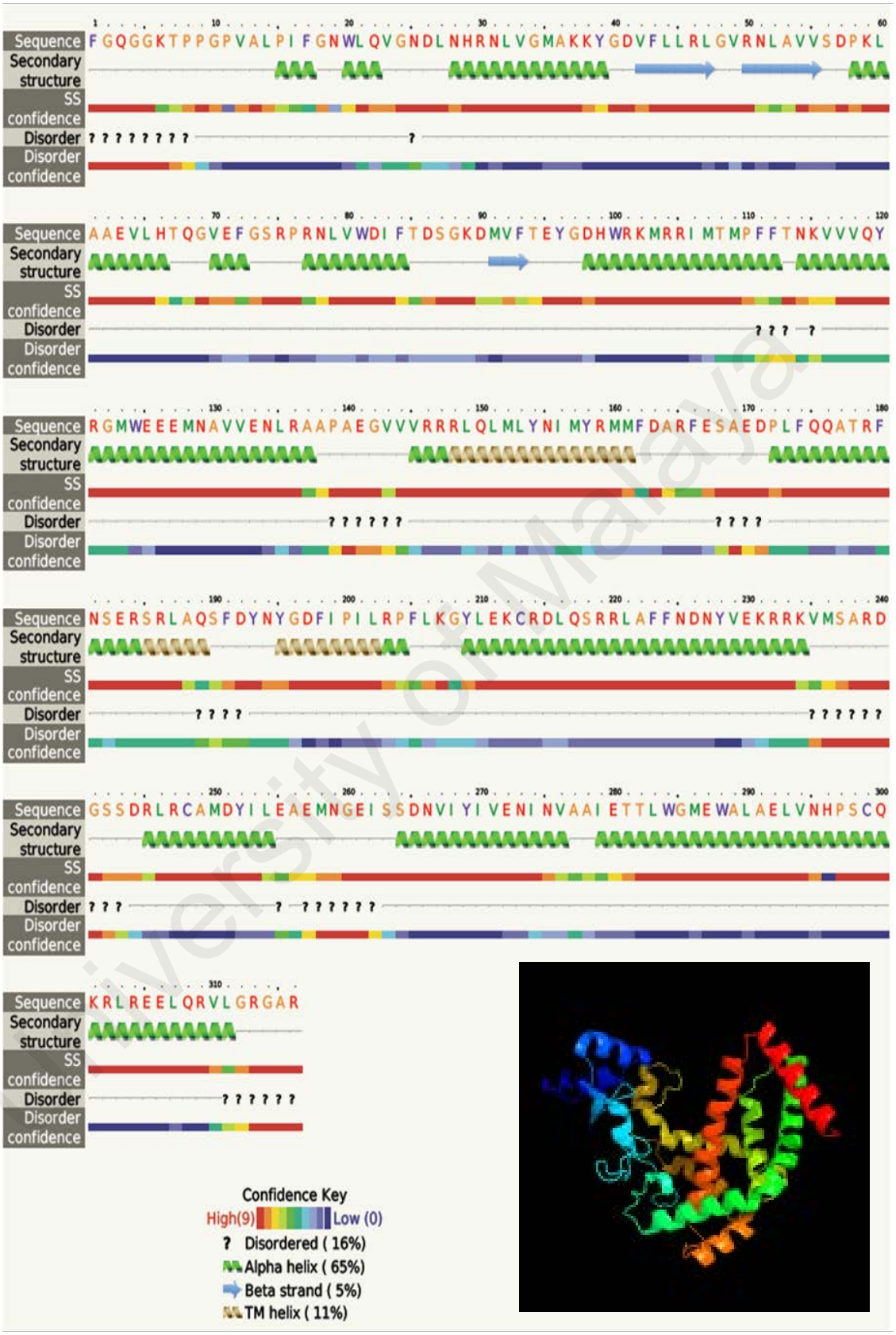


Figure 4.7: Protein secondary structure and simulated-folding of C4H1 gene sequence based on a 3D structure model of a *Arabidopsis* cytochrome P450 (insert).

Multiple sequence alignment of C4H1 with C4Hs of *Brachypodium distachyon* [Accession No: XM 003574905.1], *Hordeum vulgare* subsp. vulgare [AK371769.1], *Musa acuminata* AAA group cv. Cavendish [KF582544.1], *Musa acuminata* subsp. malaccensis [XM 009419241.1] and *Oryza sativa* Japonica [NM 001053354.1] shows that the predicted amino sequences contained the conserved domains and active residues unique to C4H proteins (Fig. 4.8).

A transmembrane binding domain was found at amino acid 9-29, common for stress-responsive proteins akin to C4H, which usually acts as cell surface receptor responsible for extracellular and intracellular communication (Whitbred and Schuler, 2000). Besides, the sequence obtained also contains the hinge motif at N terminus, the threonine-containing binding pocket motif at amino acid 270-286 and substrate recognition sites (SRS) SRS1, SRS2, SRS3 and SRS4. SRS 1, 2 and 4 are involved in interaction of the aliphatic regions of the substrates (Pina *et al*., 2012). Mutation of the amino acid Asparagine resides in SRS4 causes loss of cinnamic acid binding efficiency and reduction of the catalytic activity (Schoch *et al.*, 2003). The same group showed that the amino acid isoleucine resides in the same SRS and is responsible for substrate positioning and orientation for catalysis by site-directed mutagenesis method (Schoch *et al.*, 2003).

```
Brachypodium      --HFHPT--CRGGSMetAA----LAIRAAFAAVATSLAVYWLLNSSFLQTPNIALSLPAA
Oryza             --QPVSG--SSSSSMetAASA--MetRVA-IATGASLAVHLFVK-SFVQAQHPALTLLLP
Hordeum           QQRQAST--RAEHSMetTASASARRMetA-FAAAASLAVYWLLK-SFLHTPHPALLPAAA
C4H1              ----------------------------------------VK-FGQLPVV-ACIP
Cavendish         ----------------AASTGKLAMetLTLAA---VACTYAAKHLF-PDQSPFL-LSLP
Malaccensis       -RALATGRKPTTIAMetAASTGKLAMetLTLAA---VACTYAAKHLF-PGQSPFL-LSLP
                                                                 :

Brachypodium      AAAFVVVAIAASGPGHRSDGTPPGPAALPVLGNWLQVGNDLNHRFLARL--SARYGPVFR
Oryza             VAVFVGIAVGAKGGSGGDGKAPPGPAAVPVFGNWLQVGNDLNHRFLAAMetSARYGPVFR
Hordeum           ALVALTITLGASGK-GGGAGAPPGPAAVPVFGNWLQVGNDLNHRFLAGL--SARYGPVFR
C4H1              F--LFALPFFFVTYGGGGGKTPPGPVALPIFGNWLQVGNDLNHRNLVGMetAKKYGDVFL
Cavendish         LL-LFFLPFVFSR--SGSNGAPPGPVSFPIFGNWLQVGNDLNHRNLVDMetAKKYGNVFL
Malaccensis       LL-LFFLPFVFSR--SGSNGAVNMetAKKYGNVFL
                      . : .          :****.:.*::************** *. :  : :** **

Brachypodium      LRLGVRNLVVVSDPRLATEVLHTQGVEFGSRPRNVVFDIFTANGADMetVFTEYGDHWRR
Oryza             LRLGVRNLVVVSDPKLATEVLHTQGVEFGSRPRNVVFDIFTANGADMetVFTEYGDHWRR
Hordeum           LRLGVRNLVVVSDPRLATEVLHTQGVEFGSRPRNVVFDIFTANGADMetVFTEYGDHWRR
C4H1              LRLGVRNLAVVSDPKLAAEVLHTQGVEFGSRPRNLVWDIFTDSGKDMetVFTEYGDHWRK
Cavendish         LRLGVRNLVVVSDPKLATEVLHTQGVEFGSRPRNVVWDIFTDSGKDMetVFTEYGDHWRR
Malaccensis       LRLGVRNLVVVSDPKLATEVLHTQGVEFGSRPRNVVWDIFTDSGKDMetVFTEYGDHWRR
                  ********.*****:**:*******************:*:**** .* **************:

Brachypodium      MetRRVMetT--LPFFTARVVQQYRAMetWEAEMetDA--VVSDLRADPVARVAGVVVRR
Oryza             MetRRVMetT--LPFFTARVVQQYKAMetWEAEMetDA--VVDDVRGDAVAQGTGFVVRR
Hordeum           MetRRVMetT--LPFFTARVVQQYRAMetWEAEMetDD--VVSDLRGGSAARGPGVVVRR
C4H1              MetRRIMetTMetPFFTNKVVVQYRGMetWEEEMetNAV-----VENLRAAPAEGVVVRR
Cavendish         MetRRIMetT--LPFFTNKVVQQYRGMetWEEEMetDMetVLRDLRGDRAAQSEGIVVRR
Malaccensis       MetRRIMetT--LPFFTNKVVQQYRGMetWEEEMetDMetVLRDLRGDRAAQSEGIVVRR
                  *****:****   **** :** **:.***** ****:       :.   .*   *.****
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Brachypodium      RLQLMetLYNIMetYGMetMetFDARFESVDDPL--FVQATRFNSERSRLAQSFDYNYGD
Oryza             RLQLMetLYNIMetYRMetMetFDARFESVDDPMetFIEATRFNSERSRLAQSFEYNYGD
Hordeum           RLQLMetLYNIMetYRMetMetFDARFESVDDPMetFVEATKFNSERSRLAQSFDYNYGD
C4H1              RLQLMetLYNIMetYRMetMetFDARFESAEDPL--FQQATRFNSERSRLAQSFDYNYGD
Cavendish         RLQLMetLYNIMetYRMetMetFDARFESVSDPL--FQQATRFNSERSRLAQSFEYNYGD
Malaccensis       RLQLMetLYNIMetYRMetMetFDARFESVSDPL--FQQATRFNSERSRLAQSFEYNYGD
                  *************** ************..**: * :**:**************:*****

Brachypodium      FIPILRPFLRGYLNKCRDLQSRRLAFFNNNYVEKRRKVMetDS-PGDKDKLRCAIDHI--
Oryza             FIPILRPFLRGYLNKCRDLQSRRLAFFNNNYVEKRRKVMetDT-PGDRNKLRCAIDHI--
Hordeum           FIPILRPFLRGYLNKCRDLQTRRLAFFNSNYVEKRRKVMetDT-PGDKNKLRCAIDHI--
C4H1              FIPILRPFLKGYLEKCRDLQSRRLAFFNDNYVEKRRKVMetSARDGSSDRLRCAMetDYI
Cavendish         FIPILRPFLRSYLNKCRDLQSRRLAFFNNNYVEKRRKLMetAEREG--DRLRCAMetDYI
Malaccensis       FIPILRPFLRSYLNKCRDLQSRRLAFFNNNYVEKRRKLMetAEREG--DRLRCAMetDYI
                  *********:.**:******:*******.********:***    *  ::****::

Brachypodium      L--AAEKNGEITAENVIYIVENINVAAIETTLWSI--EWALAEVVNHPAVQTKVRGEIKD
Oryza             L--EAEKNGELTAENVIYIVENINVAAIETTLWSI--EWALAEVVNHPAVQSKVRAEIND
Hordeum           L--AAEKSGEITPENVIYIVENINVAAIETTLWSI--EWALAEVVNHPDVQRKVRGEIRD
C4H1              LEAEMetNGEISSDNVIYIVENINVAAIETTLWGMetEWALAELVNHPSCQKRLREELQR
Cavendish         LEAEMetNGEISSDNVIYIVENINVAAIETTLWSMetEWAIAELVNHPNAQTRLRKELRD
Malaccensis       LEAEMetNGEISSDNVIYIVENINVAAIETTLWSMetEWAIAELVNHPNAQTRLRKELRD
                  *   *..**:: :******************:  ***:**:****  * ::* *:.

Brachypodium      VLGDDEPITESNIQQLPYLQAVIKETLRLHSPIPLLVPHMetNLEEAKLGGYTIPRGSKV
Oryza             VLGDDEPITESSIHKLTYLQAVIKETLRLHSPIPLLVPHMetNLEEAKLGGYTIPKGSKV
Hordeum           VLGDDEPITESNISKLPYLQAVIKETLRLHSPIPLLVPHMetNLEEASLGGYTIPEGSKV
C4H1              VLGR-GARStop----------------------------------------------
Cavendish         VLGD-EPVTETNLHRLPYLQAVVKETLRLHSPIPLLVPHMetNLEEAKLGGYDIPKRTKV
Malaccensis       VLGD-EPVTETNLHRLPYLQAVVKETLRLHSPIPLLVPHMetNLEEAKLGGYDIPKRTKV
                  ***     :
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Brachypodium      VVNAWWLANNPELWEKPEEFRPERFLDEDSGVDAATIGGKADFRFLPFGVGRRSCPGIIL
Oryza             VVNAWWLANNPALWENPEEFRPERFLEKESGVDA-TVAGKVDFRFLPFGVGRRSCPGIIL
Hordeum           VVNAWWLANNPELWEKPEEFRPERFLGEESNVDA-TVGGKVDFRFLPFGVGRRSCPGIIL
C4H1              ------------------------------------------------------------
Cavendish         IVNAWWLGNNPEWWNKPEEFRPERFLDEETEVEA-LVGGKVDFRFLPFGVGRRSCPGIIL
Malaccensis       IVNAWWLGNNPEWWNKPEEFRPERFLDEETEVEA-LVGGKVDFRFLPFGVGRRSCPGIIL


Brachypodium      AMetPILALIVGKLVRSFQMetLPPPGVDKLDVSEKGGQFSLHIANHSVVAFHPIDSASt
Oryza             AL--PILALIVGKLVRSFEMetVPPPGVEKLDVSEKGGQFSLHIAKHSVVAFHPISASto
Hordeum           AL--PILALIVGKLVRSFEMetVPPPGVDKLDVSEKGGQFSLHIANHSLVAFHPISASto
C4H1              ------------------------------------------------------------
Cavendish         AL--PLLGLIVGKLVKEFEMetVPPPGTDKIDVTEKGGQFSLQIAEHSTIAFHPIAPSto
Malaccensis       AL--PLLGLIVGKLVKEFEMetVPPPGTDKIDVTEKGGQFSLQIAEHSTIAFHPIAPSto


Brachypodium      op
Oryza             p-
Hordeum           p-
C4H1              --
Cavendish         p-
Malaccensis       p-
```

Figure 4.8: Multiple sequence alignment of C4H1 amino acid sequence with C4Hs from other plant species. The completely identical amino acids are indicated with asterisks (*). Conserved domains such as hinge motifs, T binding pockets are highlighted in colors. Substrate recognition sites (SRS) are in rectangles and the haem binding domains are underlined.

Sequence identity of 75% or more with a functionally identified ortholog is often considerate as a sufficient criterion for the annotation to a newly isolated gene (Jiang *et al.*, 2006). Although this has frequently has been acceptable, several cases of uncertainty exist. For instance, many structurally closely related proteins within the cytochrome P450 family have widely differing functional properties, and even the exchange of only three amino acid residues is responsible for substrate specificity and substitution (Lindberg and Negishi, 1989). On the other hand, P450 proteins with only 26% sequence identity may catalyze the same biochemical reaction (Ma *et al*., 1994). Therefore, it is important to identify and characterize the functionality of the isolated *C4H1* gene. One of the methods would be the demonstration *in vitro* of its exclusive hydroxylation of cinnamate in the *para* position of the aromatic ring to give 4-coumarate and thus giving supporting evidence for its' specific role in the phenylpropanoid metabolism. Nevertheless, the best way to prove and confirm the number of genes or members in an enzyme superfamily is to analyze at the level of genomic complexity.

Koopmann *et al.* (1999) isolated a single *C4H* gene encoding the mRNA and protein in parsley and further demonstrated its *in vivo* cell-type-specific distribution patterns and PAL-C4H coordination without interference from structurally similar gene products using immunotitration, *in situ* mRNA and protein localization methods. However, the authors could not definitely exclude the existence of a paralog protein which has similar function but is structurally dissimilar. Hence, the authors suggested that the latter possibility can be tested using defined mutants which supports our approach of generating a C4H1 knock-down/ knock-out culture of *B. rotunda*.

4.4.2 RNAi vector construction and introduction into *Agrobacterium*

pANDA vector carrying partial C4H hpRNA

Partial C4H1 cDNA isolated from the previous experiment was cloned into the RNAi vector, pANDA, and the resulting plasmid was named pANDA-C4H1 henceforth. PCR analysis confirmed the presence of *C4H1* gene sequences (≈ 700 bp) and *gusl* gene (≈ 670 bp) in the *A. tumefaciens* LBA4404 transformed with the plasmid (Fig. 4.9a).

The formation of bright yellow coloration around the bacterial conlonies in Benedict's test confirmed the *Agrobacterium* (Fig. 4.9b). Bright yellow colouration, of cuprous oxide, forms in the presence of 3-ketolactose resulted from lactose oxidation, a reaction carried out by *Agrobacterium* in Benedict's reagent (Bernaerts and De Ley, 1963). This simple method was performed to eliminate the cross-contamination possibility of *E. coli* which extensively used in plasmid cloning procedures.

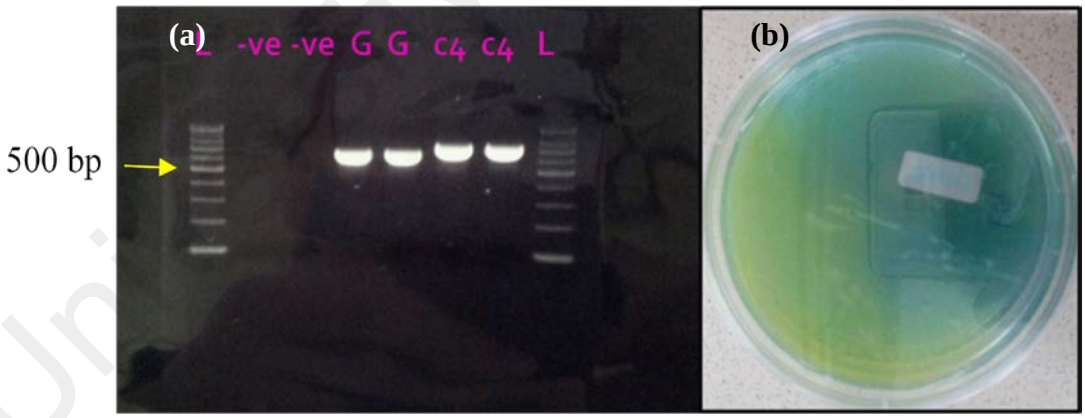


Figure 4.9: Confirmation of *Agrobacteria* carrying RNAi vector, pANDA-C4H1. (a). Agarose gel PCR products for confirmation of *Agrobacterium* carrying pANDA–C4H1. L = 100 bp DNA ladder, -ve = negative control, G = *gusl*, C4 = C4H1. (b). Benedict's test of *Agrobacterium* carrying pANDA–C4H1. Bright yellow colonies formation confirmed the presence of *Agrobacteria*.

4.4.3 Transformation of *B. rotunda* cell suspension cultures with *Agrobacterium* carrying RNAi vector

Cells recovered from stringent hygromycin selection (LD_{75}) after transforming with *Agrobacterium* carrying pANDA–C4H1 on media plate were observed under the microscope (Fig. 4.10). Untransformed cells appeared bleached and had retarded to growth, as indicated with arrows in figure 4.10. The hygromycin-resistant calli were picked up carefully under the microscope and transferred into liquid media containing hygromycin for future propagation. Single callus was propagated in isolate to ensure homogenous population regeneration. Each calli regenerated was considered as single transformation event generated and thus resulted in a total number of 93 independent transformation events. PCR analysis on the hygromycin resistant cells using primers specific to *gusI* gene (which resides in between C4H1 inverted repeat inside the T-DNA) revealed eleven transgenic lines were obtained, and this result suggested an approximate 11.83% transformation efficiency. The presence of *gusI* gene of ≈ 630 bp indicated successful transformation events in the cell lines L3, L5, L7, L8, PD, C2, C5, C7, C9, C10 and C12 (Figs. 4.11 & Fig. 4.12).

Failure of detection of the transgene in some hygromycin-resistant cell lines could be a result of transient expression of the resistance gene of unintegrated copies of the T-DNA or chimerism in the transformants (Goetz *et al*., 2012; Mohamed *et al*., 2010). Alternative methods are usually performed to verify the transgene genomic integration in the transgenic obtained (Flachowsky *et al.*, 2008). In this case, Southern hybridization was performed on the transgenic cell lines L8, and PD using *gusI* probe. These cell lines were picked for the analysis as representative because of good growth performance in liquid media. Southern analysis confirmed the transgene integration and, the gene copy number was found to be two. As anticipated, no hybridisation signal was observed in the non-transformed control cell lines (Fig. 4.13).

Figure 4.10: Cells recovered from hygromycin selection after transforming with *Agrobacterium* carrying pANDA-C4H1. Black arrow indicates dead or growth-retarded cells while yellow arrow indicates hygromycin-resistant cells developed after stringent selection.

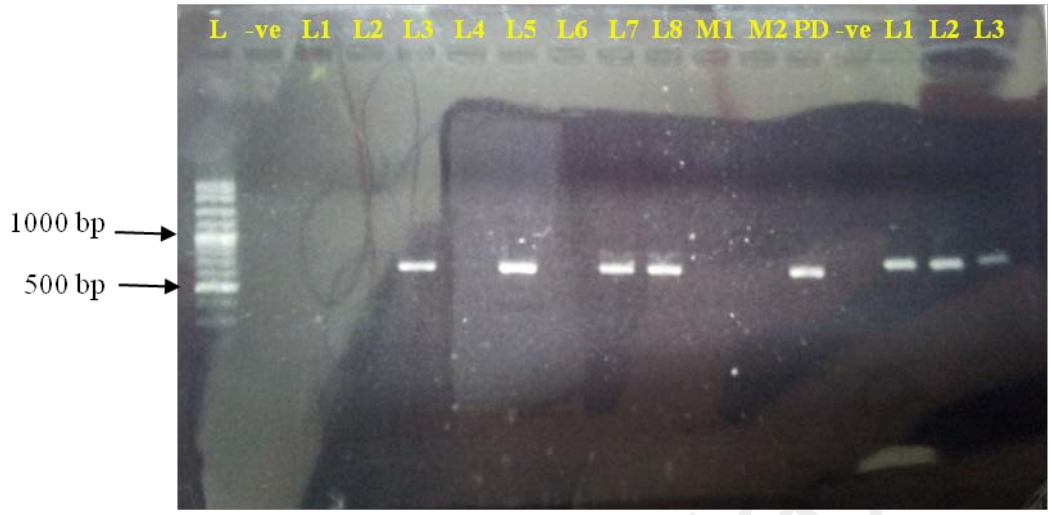


Figure 4.11: PCR analysis on transformed cell lines L1 – 8, M1, M2 and PD using primers specific to *gusI* gene and L1, L2 and L3 using primers specific to endogenous C4H gene as an internal control. L represents 100bp DNA ladder and –ve is a negative, no template control.

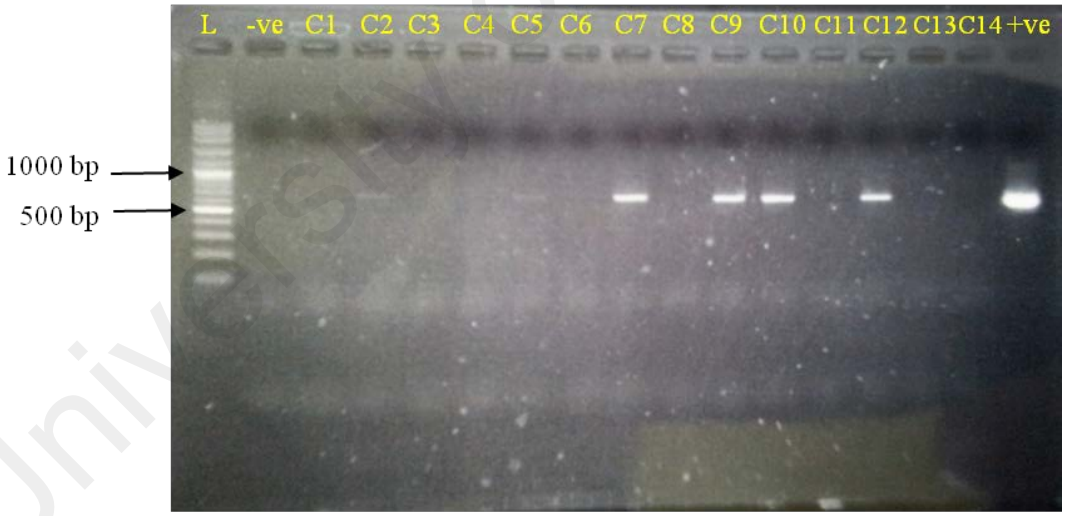


Figure 4.12: Gel picture showing the results of PCR analysis on transformed cell lines C1 – 12 using primers specific to *gusI* gene. L represents 100bp DNA ladder, –ve is a negative, no template control and +ve is a positive control using pANDA-C4H vector as PCR template.

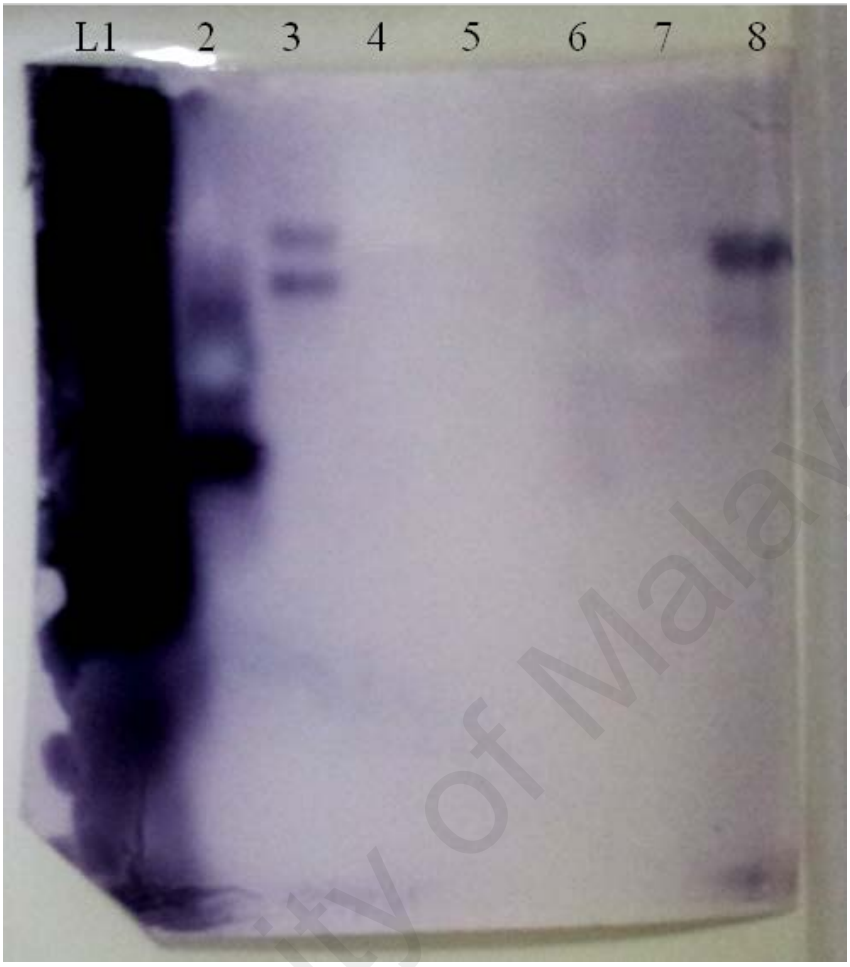


Figure 4.13: Southern hybridisation. L1 = technical control (biotin-labelled DNA provided in the kit), 2 = *gusI* gene fragment (PCR product), 3 = knockdown cell suspension line L8, 4 = PD, 5 = blank, 6 and 7 = negative control (wild type cell suspension), 8 = pANDA-C4H plasmid control.

4.4.4 Effects of C4H dsRNA on the expression of C4H

The transgenic cell suspension culture line L8, which showed positive results in PCR and stable integration in Southern analysis was maintained in selection media up to three months for further analysis. To examine the RNAi influence of the dsRNA introduced into the cells, C4H1 transcript levels were determined. Quantitative RT-PCR analysis revealed that the C4H1 gene expression in the transgenic cell lines was suppressed when compared to wild type (Fig. 4.14). The results showed a significant reduction in C4H1 gene expression in L8 cell transformed with the RNAi construct, with 0.75-fold differences compared to the wild type cell suspension. Relative quantification of the gene expression was normalised with *B. rotunda* endogenous β-actin gene expression and calibrated with no-template control (NTC). Nevertherless, in order to examine whether the knockdown effects affects onto other pathways, global expression profile could be established via transcriptomes analysis and comparison between the knockdown and wild type cells could be carried out.

To test whether the reduction in C4H1 transcripts resulted from the suppression effect of C4H1 dsRNA, small RNA was extracted and subjected to northern hybridisation analysis using probes specific to C4H1. Short (≈ 22 nt) C4H1-derived RNAs was detected in the blot (Fig. 4.15). Generation of silencing-associated small interfering RNA species complementary to the silenced gene has been detected in many RNAi studies such as in petunia (Metzlaff *et al.*, 1997; Napoli *et al*., 2000), and rice (Goto *et al*., 2003; Miki and Shimamoto, 2004), which indicates the occurrence of RNA silencing in the cells. Thus, the result supported that the reduction of the C4H1 transcript level in L8 cell line was attributed to the silencing effects of C4H1 dsRNA.

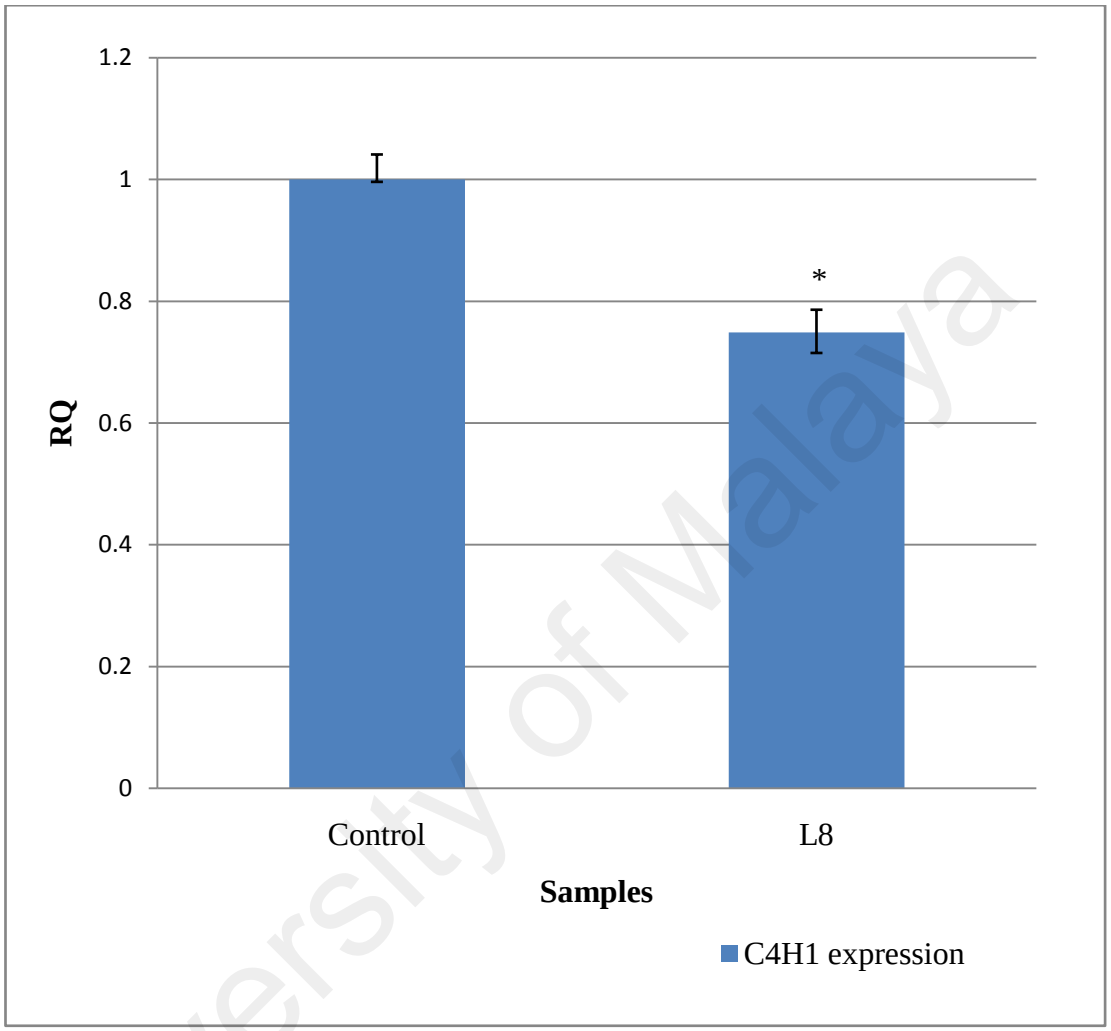


Figure 4.14: Quantitative RT-PCR analysis of C4H1 transcript levels in wild type and L8 transgenic cells harbouring C4H1 inverted repeat transgene. Bars represent C4H1 transcript levels and error bars represent standard deviation with n = 4. Asterisk mark indicates statistical significance when means were compared with using T-test at a critical t-value of $p < 0.05$.

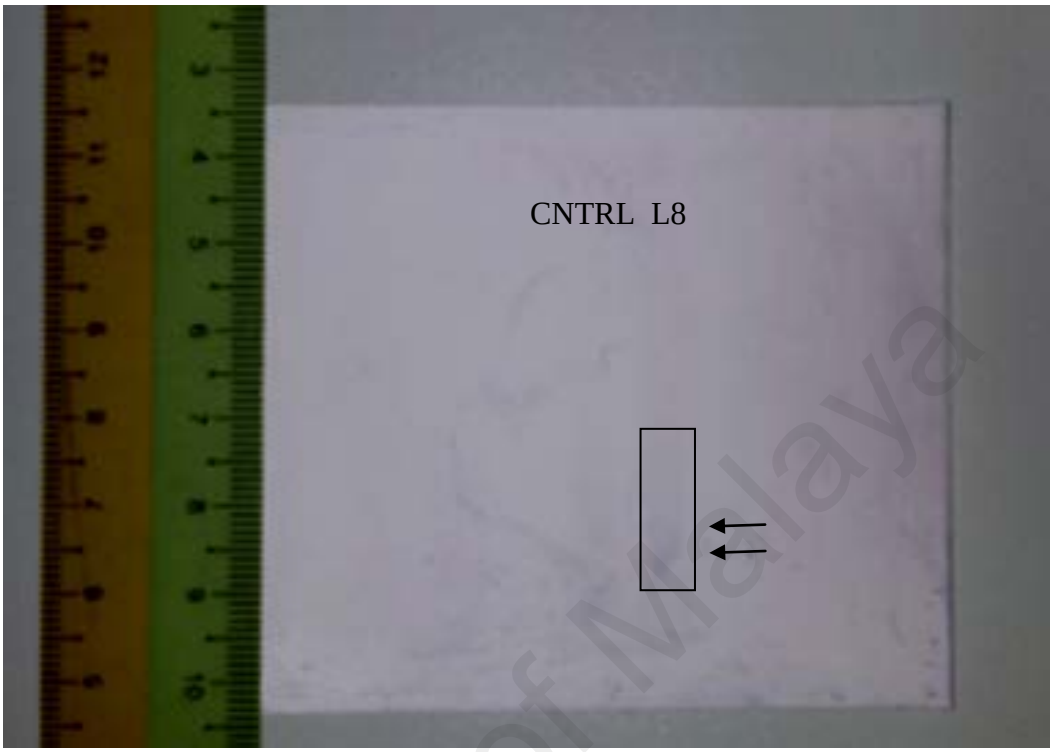


Figure 4.15: Northern hybridisation analysis using probe specific to partial C4H1 gene fragments. Intensive small RNA homologous to the probes was detected in RNAi silencing cell line L8 (Lane L8, indicated in with arrows in box) while absent in Control (Lane CNTRL) wild type cells indicates the occurrence of specific RNA degradation.

4.4.5 Effects of C4H dsRNA on primary metabolites

To evaluate the C4H dsRNA effects on the metabolite profiles, the RNAi cell line L8 and wild type cell suspension were subjected to LC-MS analysis. Table 4.11 summarized the changes in primary metabolites and their profile in the cell suspension cultures. The RNAi cell line possesses a similar profile of primary metabolites to the wild type control, indicated by the relative abundance of each primary metabolite in different colour codes (Table 4.11). As a proportion of total primary metabolite comprising of amino acids, organic acids, polyamines, and simple sugar phosphates, citric acid was shown to be the most abundance primary metabolite, accounting for 92% of the total primary metabolites measured, while the sugar phosphates are the least abundant primary metabolites in the suspension cell cultures. Some of the phosphate sugars content, e.g. erythrose-4-phospate and glucose-1-phosphate/ glucose-6-phosphate found in the suspension were almost negligable. Meanwhile, lysine, valine, glutamine, histidine, arginine, Leucine/ Isoleucine and tryptophan are the major amino acids found in the cell suspension.

Table 4.11: LC-MS analysis of the primary metabolite profiles in RNAi cell line L8 and wild type cell suspension cultures.

Primary metabolites	Groups/ Classifications	Relative abundance ± SD		Fold change
		Wild Type	L8 (RNAi cell line)	
Glycine	AA	5.09 ± 0.02	3.20 ± 0.01	-0.68
Homeserine	AA	4138.20 ± 18.06	4383.66 ± 6.16	0.08
Glutamine	AA	16549.40 ± 34.24	91955.79 ±160.29	2.47*
Histidine	AA	22703.44 ±103.50	41166.05 ± 21.12	0.86
Arginine	AA	63743.91 ± 178.43	129468.36 ± 88.34	1.02*
Alanine	AA	256.60 ± 0.44	448.19 ± 0.12	0.80*
Asparagine	AA	1058.75 ± 2.84	472.29 ± 1.53	-1.16
Aspatic acid	AA	6234.85 ± 17.64	2666.03 ± 10.21	-1.23
GABA (gamma-aminobutyric acid)	AA	3016.92 ± 8.87	2537.83 ±2.44	-0.25
Glutamic acid	AA	740.67 ± 0.16	906.01 ± 0.27	0.29*
Citrulline	AA	2623.34 ± 8.02	5703.51 ± 2.80	1.12*
Proline	AA	141.43 ± 0.26	181.40 ± 0.09	0.36
Ornithine	AA	1137.15 ± 5.63	1088.24 ± 4.88	-0.06
Phenylalanine	AA	13.97 ± 0.05	9.03 ± 0.01	-0.63
Cytosine	AA	223.31 ± 0.19	284.33 ± 0.13	0.35*
Adenine	AA	1783.52 ± 13.15	1865.88 ± 3.04	0.07
Guanine	AA	1673.93 ± 7.60	2357.17 ± 1.46	0.49
Thymine	AA	525.41 ± 2.39	727.27 ± 0.51	0.47

Table 4.11 Continued

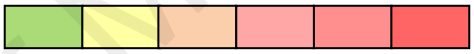
Valine	AA	15774.20 ± 95.13	17112.41 ± 23.36	0.12
Tyrosine	AA	5196.74 ± 37.52	5471.69 ± 8.23	0.07
Tryptophan	AA	16616.66 ± 132.42	43437.26 ± 20.24	1.39*
Hydroxyproline	AA	509.00 ± 3.79	392.90 ± 1.36	-0.37
Leucine/ Isoleucine	AA	21705.03 ± 149.44	23001.78 ± 62.35	0.08
Lysine	AA	19835.85 ± 36.82	114872.39 ± 197.70	2.53*
Methionine	AA	26.50 ± 0.23	51.27 ± 0.04	0.95
Uracil	AA	273.20 ± 0.66	172.41 ± 0.14	-0.66
Carnosine	AA	62.00 ± 0.31	100.51 ± 0.06	0.70
Putresine	Polyamine	136.71 ± 0.17	387.43 ± 0.35	1.50*
Caffeic acid	OrgA	33.14 ± 0.02	197.14 ± 0.68	2.57*
Antranilate	OrgA	1207.40 ± 5.31	261.88 ± 2.19	-2.20
Creatine	OrgA	8.87 ± 0.01	12.17 ± 0.01	0.46
Malic acid	OrgA	1014934.53 ± 1118.51	1919004.56 ± 2164.54	0.92*
Lactic acid	OrgA	1109.19 ± 1.05	2073.66 ± 1.24	0.90*
Gluconic acid	OrgA	67380.13 ± 118.18	119149.10 ± 202.63	0.82
2-Oxoisovaleric acid	OrgA	87944.18 ± 92.78	141672.15 ± 180.45	0.69*
cis-Aconitic acid	OrgA	1048998.58 ± 7895.53	497468.56 ± 1045.58	-1.08
Citric acid	OrgA	32648101.08 ± 117779.53	33533844.75 ± 19805.61	0.04
Oxaloacetic acid	OrgA	3426.93 ± 12.89	10935.04 ± 34.35	1.67
Shikimic acid	OrgA	183.61 ± 0.72	206.05 ± 0.31	0.17
2-Oxoglutaric acid	OrgA	395.99 ± 0.31	214.26 ± 0.48	-0.89*

Table 4.11 Continued

Isocitric acid	OrgA	511718.70 ± 1750.74	518364.51 ± 321.80	0.02
Glyoxlic acid	OrgA	357.39 ± 0.72	688.00 ± 0.88	0.94*
Glycolic acid	OrgA	30.44 ± 0.10	45.96 ± 0.08	0.59
Oxalic acid	OrgA	170.57 ± 0.06	312.00 ± 0.39	0.87*
Shikimic-3-phosphate	OrgA	0.20 ± 0.00	0.08 ± 0.00	-1.39*
6-phosphogluconic acid	OrgA	0.07 ± 0.00	0.08 ± 0.00	0.21
Ribose-5-phosphate	POA	1.79 ± 0.01	2.93 ± 0.02	0.71
3-phosphoglyceric acid	POA	2.17 ± 0.00	2.76 ± 0.02	0.34
Glycerol-3-phosphate	POA	4.62 ± 0.01	5.31 ± 0.01	0.20
S-adenosyl methionine	Polyamine	15.71 ± 0.05	11.33 ± 0.01	-0.47
Spermine	Polyamine	85.59 ± 0.30	102.93 ± 0.07	0.27
Spermidine	Polyamine	82.22 ± 0.98	202.65 ± 0.81	1.30
Erythrose-4-phosphate	Sugar P	0.03 ± 0.00	0.03 ± 0.00	-0.14
Glucose-1-phosphate/ Glucose-6-phosphate	Sugar P	0.01 ± 0.00	0.00 ± 0.00	-0.70
Xylulose-5-phosphate	Sugar P	0.70 ± 0.00	0.87 ± 0.00	0.32

Table 4.11 Continued

Ribulose-5-phosphate	Sugar P	1.63 ± 0.00	2.89 ± 0.00	0.83
Fructose-6-phosphate	Sugar P	2.01 ± 0.01	1.67 ± 0.01	-0.26
Fructose-1,6-phosphate	Sugar P	0.25 ± 0.00	0.31 ± 0.00	0.29
Glutathione (red)		1.69 ± 0.00	0.88 ± 0.01	-0.94*
Glutathione (ox)		70.17 ± 0.19	34.20 ± 0.43	-1.04

Most abundance > Least abundance 

Statistical significance was indicated by asterisks sign (*) where the relative abundance of the metabolite was contrasted using Student T-test at a critical t-value of p = 0.05. AA = Amino acids, OrgA = Organic acids, and Sugar P = Sugar phosphates.

Similar primary metabolites profile in general between RNAi cell line L8 and wild type control cell suspension suggested that the C4H dsRNA caused no or minimal off-targets effect. Nevertheless, the data show significant changes in the production of several primary metabolites when C4H1 expression was suppressed in L8 trangenic cell suspension (Figs. 4.16 & 4.17).

Surprisingly, significant increment was observed for glutamine, arginine, alanine, glutamic acid, citrulline, cytosine, tryptophan, lysine, putresine, caffeic acid, malic acid, lactic acid, 2-Oxoisovaleric acid and glyoxlic acid. While, significant reduction is only observed in 2-Oxoglutaric acid and shikimic-3-phosphate. The relative quantification of the phenolic acids, coumaric acid and cinnamic acid is shown in figure 4.18. The concentration of cinnamic acid in the RNAi cell line L8 was 4-fold lower than the control wild type cell suspension while the coumaric acid concentration remained low in both cells.

Polyamines are organic polycations that stimulate DNA replication, transcription and translation, and interact with phytochrome and hormones in plant cell in response to various environmental factors and developmental stages (Bitrián *et al.*, 2012). The biosynthesis of many polyamines is regulated by anabolic and catabolic processes, also by the conjugation of their hydroxycinnamic acid amides (Alcázar *et al.*, 2010). Thus, the availability of the hydroxycinnamic acids could affect the concentration of the polyamines and regulate their biosynthesis and catabolism (Martin-Tanguy, 2006).

Significant increment in free Putrescine in C4H1-downregulated cell suspension could be due to the reduction of hydroxycinnamate acid in the cell (Fig. 4.17a). This is again supports that the enzyme C4H1 downregulated functions at the second step in the phenylpropanoid pathway which was also suggested by Dixon (2005). Whether the enzymes involved in the biosynthesis of Putrescine such as arginine decarboxylase

(ADC) or ornithine decarboxylase (Alcázar *et al.*, 2005) are involved, could be revealed by detailed transcriptome analysis.

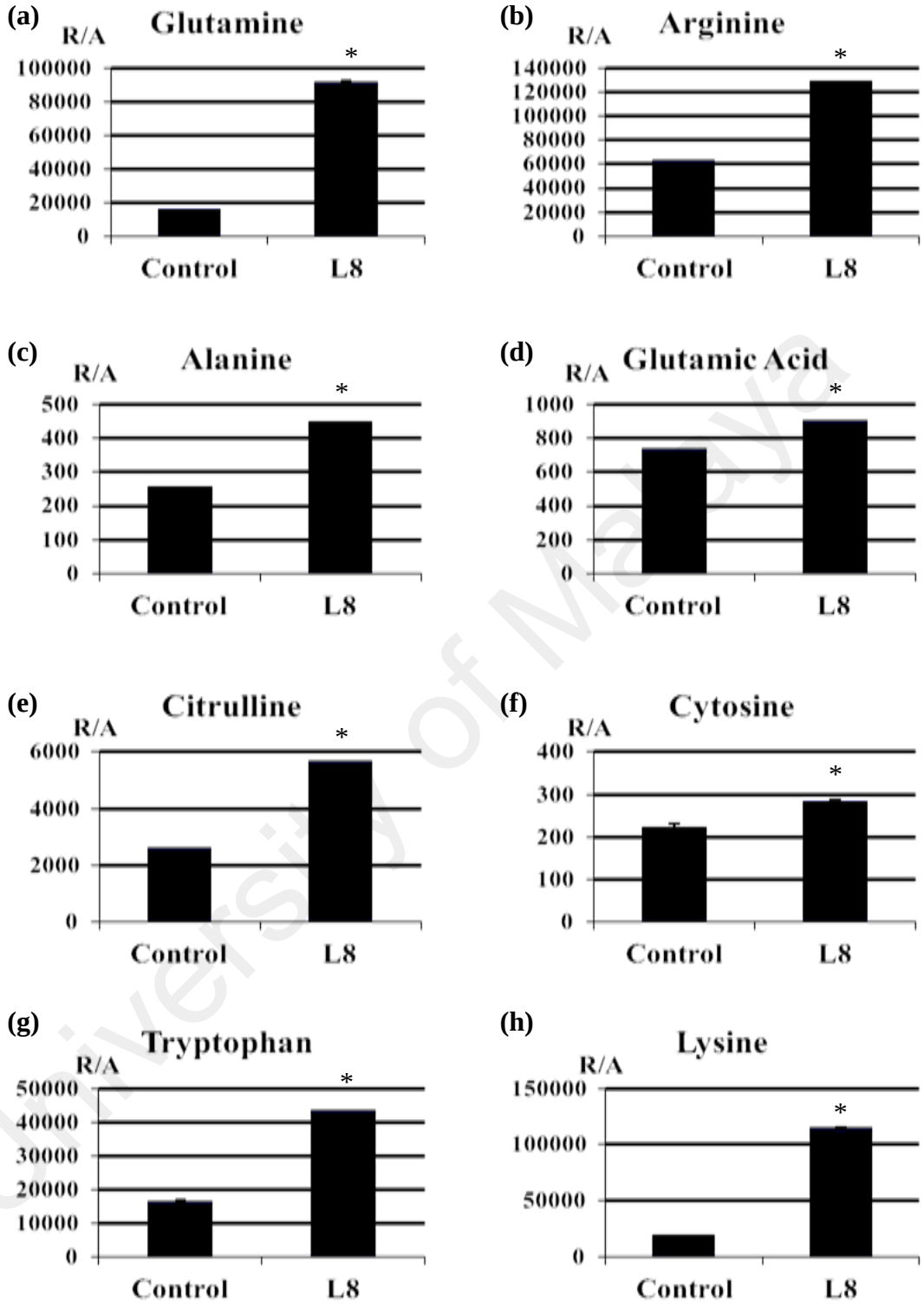


Figure 4.16: Relative abundance (R/A) of the differentially regulated amino acids: (a) glutamine, (b) arginine, (c) alanine, (d) glutamic acid, (e) citrulline, (f) cytosine, (g) tryptophan, (h) lysine in the RNAi cell line L8 and wild type non-transformed cell suspension culture. Error bars indicate SD where n = 9. Asterisks indicate significant statistical difference in T-test at a critical t-value of *p*=0.05.

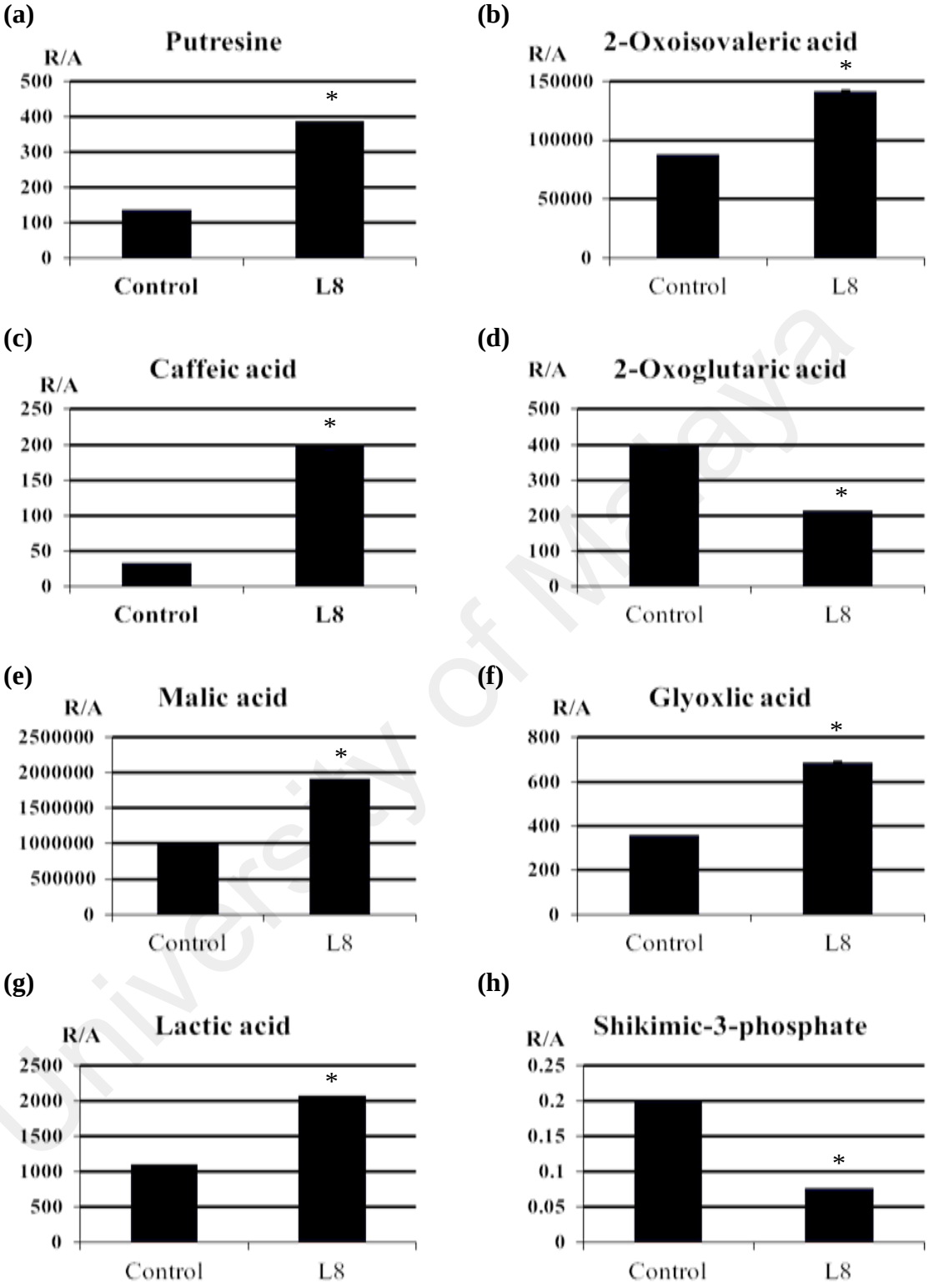


Figure 4.17: Relative abundance (R/A) of the differentially regulated polyamine and organic acids: (a) putresine, (b) 2-Oxoisovaleric acid, (c) caffeic acid, (d) 2-Oxoglutaric acid, (e) malic acid, (f) glyoxlic acid, (g) lactic acid, and (h) shikimic-3-phosphate in the RNAi cell line L8 and wild type non-transformed cell suspension culture. Error bars indicate SD where n = 9.

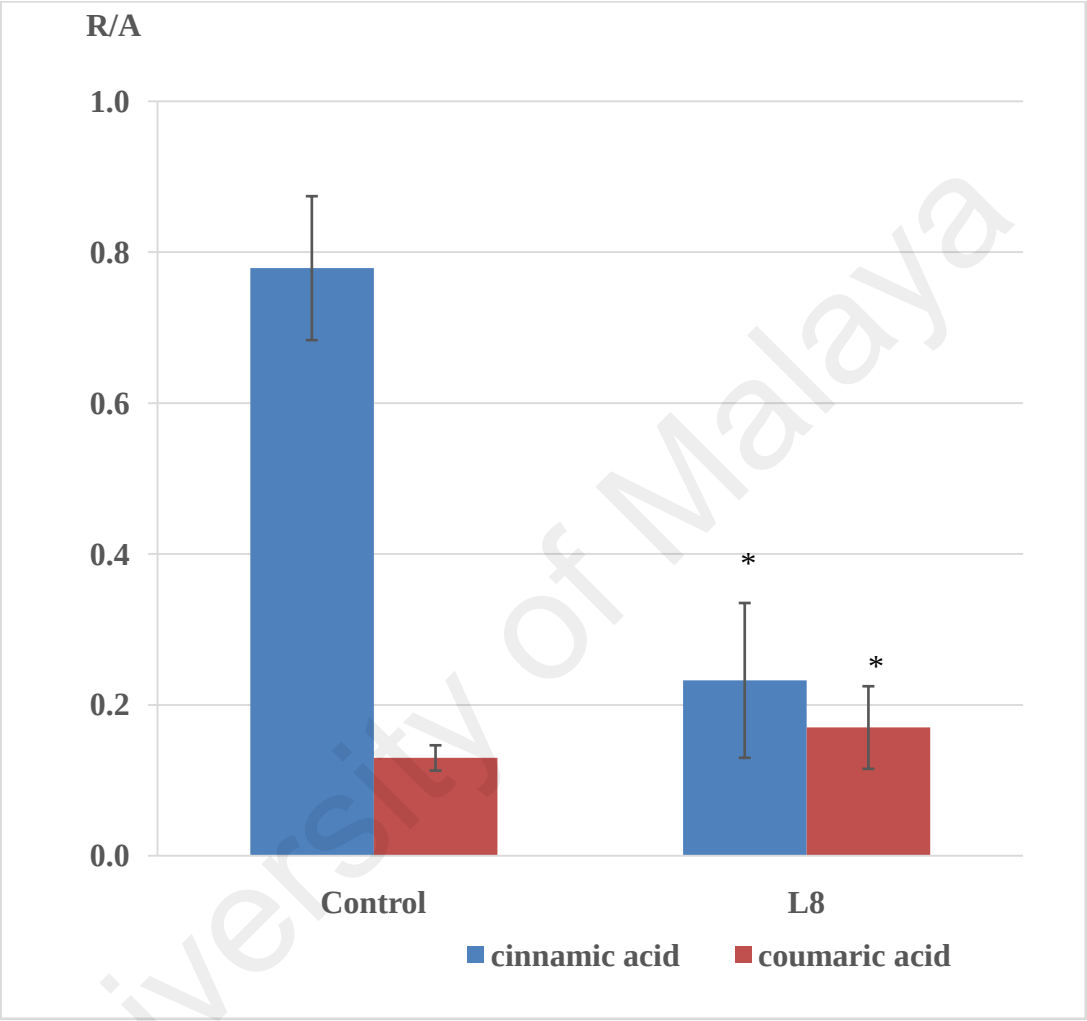


Figure 4.18: Relative abundance (R/A) of the cinnamic acid and coumaric acid concentration in the RNAi cell line L8 and wild type control cell suspension culture. Error bars indicate SD where n = 9. Asterisk indicates significant statistical difference at a critical t-value of *p* = 0.05 contrasted by T-test.

Significant increment of ≈ 50-fold was observed for caffeic acid in L8 C4H1-knockdown cell line when the concentration was compared to control (Fig. 4.17c). Interestingly, suppression of the enzyme C4H1 using dsRNA method in *B. rotunda* cell suspension has led to an increment of this compound. Caffeic acid is an organic acid consist of a phenolic ring and acrylic functional group like cinnamic acid and coumaric acid (Fig. 4.19). The compound itself and its' derivatives Caffeic acid phenethyl ester (CAPE) has been shown to be a potent antioxidant present in low density lipoprotein α-tocopherol (Gülęin, 2006). CAPE is also a very important antiproliferative and antitumor agent widely studied in anticancer research (Akyol *et al.*, 2012; Pramanik *et al.*, 2012).

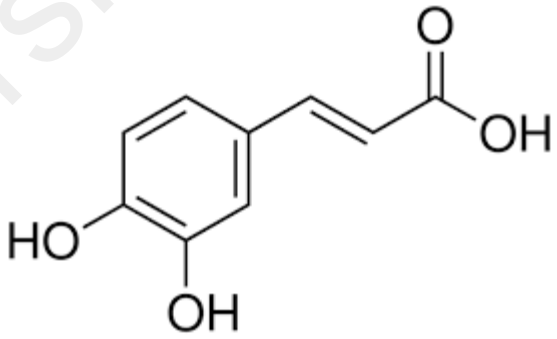


Figure 4.19: Chemical structure of Caffeic acid (3, 4-dihydroxycinnamic acid).

(Akyol *et al.*, 2012)

4.4.6 Effects of C4H dsRNA on secondary metabolites production

When the secondary metabolites production was screened via LC-MS, pinostrobin and alpinetin were measured at a detectable level (Fig. 4.20). When their percentages of dry extract in the RNAi cell line L8 was compared with the wild type control, significant reduction in concentration was observed.

When the enzyme C4H was downregulated, the pathway might be diverted into monoligol biosynthesis where an increase in the caffeic acid was observed (Fig. 4.17c), which makes the knockdown culture useful for production of the precursor for CAPE (Renouard, 2014).

The results suggested that C4H1 is important in biosynthesis of pinostrobin and alpinetin wherein knockdown of the expression caused reduction in the compounds production. However, we couldn't rule out the possibility of the effects of the C4H1 dsRNA on other isomer forms of C4H or their homologs, which might be also involved in phenylpropanoid pathway. RNAi suppression of a Class III 4-Coumarate:CoA Ligase (4CL) altered the flavonoid derivatives contents in *Pinus taeda* bark, was presumably depends on a distinct 4CL member (Wagner *et al.*, 2009).

The availability of the genome sequence *of B. rotunda* would be useful to better screening of metabolic engineering targets as well as the effects of the knockdown effects of C4H1 to their gene family members. Besides, transcriptome analysis could be carried out on the knockdown cell to evaluate the phenylpropanoid pathway-related gene expression profile and elucidation of the pathway biosynthesis flux (Md-Mustafa *et al.*, 2014).

Nevertheless, knockdown of C4H1 has provided information about its' function in primary and secondary metabolites production without affecting the cell growth. The

system developed would be useful for further exploring gene silencing endeavour in *B. rotunda* cell suspension culture. Although the compound production is low, elicitation or substrate feeding could be performed in the knockdown cell suspension culture in order to enhance the compound production which usually occurs during stress response (Sivanandhan *et al.*, 2014; Verma *et al.*, 2014) or organ structure establishment (Asano *et al.*, 2013).

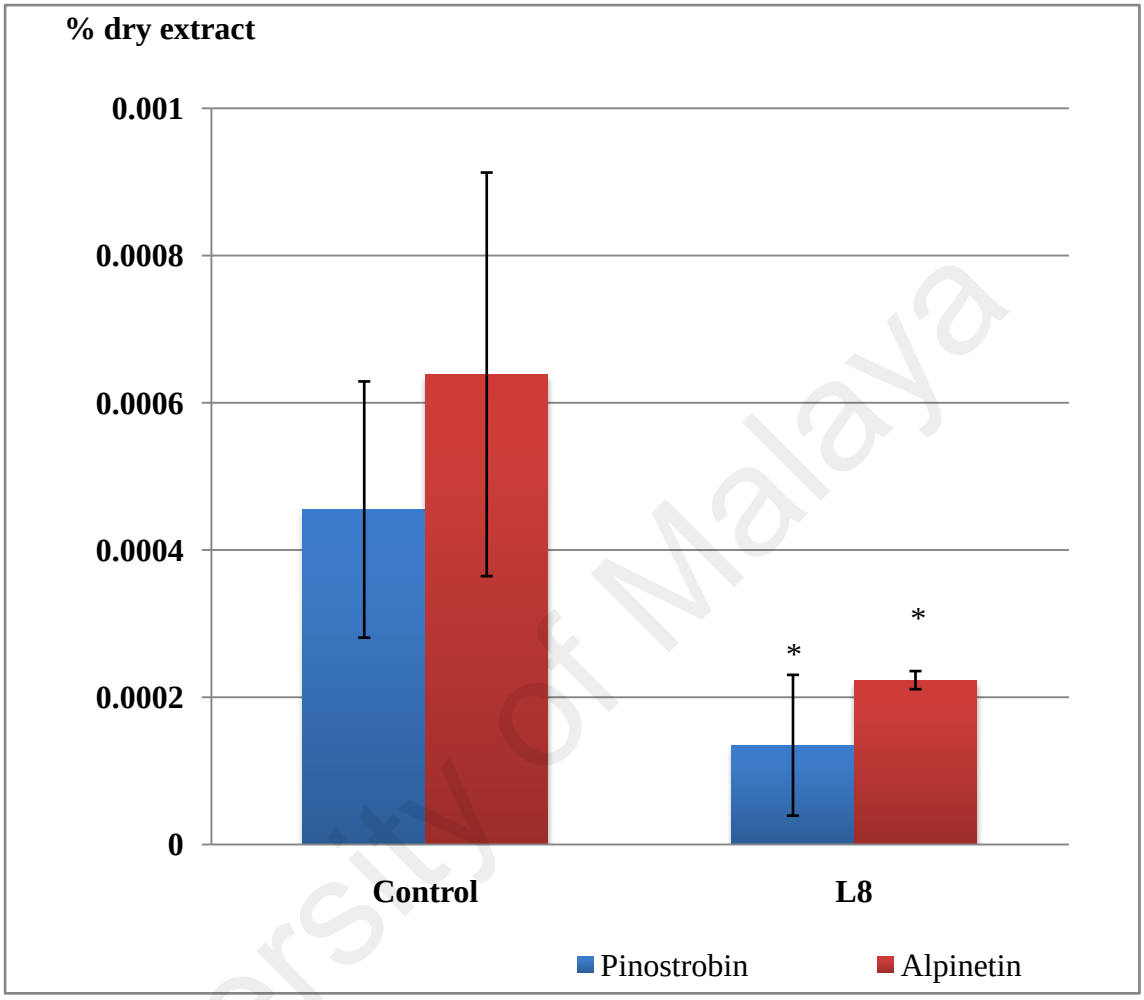


Figure 4.20: The secondary metabolites production in knockdown *B. rotunda* cell culture as compared to the wild type Control. Asterisk indicates significant statistical difference at a critical t-value of $p = 0.05$ contrasted by T-test.

CHAPTER 5: GENERAL DISCUSSION

Extracts of *B. rotunda* contain a number of valuable pharmacologically important bioactive compounds, which are mostly cyclohexenyl chalcone derivatives (CCD), flavones and flavanoids. These compounds have been shown to exhibit appreciable anti-Dengue protease activities, especially the CCDs such as panduratin A. However, CCDs are not the major compound found in the extract. In order to gain insight into the phenylpropanoid biosynthesis pathway which is responsible for producing these compounds, RNAi/ knockdown of the enzyme, cinnamate-4-hydroxylase (C4H) involved in the second switch point of the pathway was demonstrated.

In order to evaluate the knockdown effect on the enzyme C4H in *B. rotunda*, in this study, *Agrobacterium*-mediated transformation of *B. rotunda* cell suspension culture was developed. Regeneration of the suspension cell culture via somatic embryogenesis was also performed to resolve the problems of recalcitrance to regeneration in *B. rotunda*. The success of the somatic embryogenesis protocol also enabled the generation of transgenic *B. rotunda* cell culture with a low rate of chimerism, a problem which is usually associated with transgenic plant research. However, the long timeframe of the regeneration period limited the assessment of the knockdown effect to working at a cell suspension level instead of with regenerated whole plants, and this limited the detection of the compounds as some are only produced during organ structure establishment such as in rhizomes, stems and roots. In this case, organ culture could be established to overcome this problem, or a combination of genetic modification and elicitor treatment to enhance target secondary metabolites.

Besides, three isomers of cinnamate-4-hydroxylase (C4H), C4H1, C4H7 and C4H9 have been identified. C4H1, which shows homology and high similarity with others plants' C4H was chosen as the target for the knockdown study. Knockdown of C4H1 gene expression was confirmed by quantitative Real-Time PCR and northern blotting experiments. Evaluation of the primary metabolite profiles revealed that the knockdown had no detrimental effect on the cell suspension and was associated with an unusually high level of caffeic acid production, which could be useful to produce the pharmaceutically important compound, caffeic acid phenethyl ester (CAPE) (Omene *et al.*, 2015).

On the other hand, knockdown of C4H1 lead to a significant reduction in the level of secondary metabolites, particularly in pinostrobin and alpinetin production. The results supported the involvement of C4H1 in the biosynthesis pathway and suggested that overexpression of this isomer could enhance the compound production or could produce useful intermediate products, which could be useful for obtaining panduratin A through a biotransformation process.

Nevertheless, the availability of a *B. rotunda* genome sequence would be advantageous to support RNAi silencing studies involving genes/enzymes from different gene families (Dang *et al.*, 2013). This would facilitate pathway elucidation and the mining of metabolic engineering targets for compound enhancement. Therefore, further studies should be carried out to examine if knockdown of C4H1 influences the expression level or functionality of other C4H enzyme isomers. Additionally, evaluation of compound production in regenerated whole plants of the knockdown transformants should be carried out to better understand the enzyme function in different developmental stages and organs such as in rhizome and roots.

CHAPTER 6: CONCLUSIONS

In this study, a reliable cell suspension culture system via somatic embryogenesis for the fingeroot ginger, *B. rotunda* has been developed. Regeneration via somatic embryogenesis has been found at a high frequency of 1,433.33 ± 387.87 somatic embryos per ml settled cell volume on plant growth regulator free media plate with about half (53.5 ± 7.9%) of the somatic embryos developing into complete plantlets.

A protocol for the *Agrobacterium*-mediated transformation of *B. rotunda* cell suspension was also developed and optimised. Efficient transformation efficiency was achieved when the cells were infected with *Agrobacterium* for 10 min and co-cultivated for 2 days. Study of the natural tolerance of the cell suspension cultures and minimal inhibitory effects towards the selection agent, hygromycin B in liquid media and in solid media, showed that *B. rotunda* cell suspension was more sensitive to the selection agent in liquid media compared to solid agar media. Thus, a lower concentration of selection agent (i.e. 10 mgL^{-1}) should be applied in liquid media while a higher concentration (i.e. 25 mgL^{-1}) should be used with solid media.

Partial gene sequences for three isomers of C4H enzyme were cloned in this project. C4H1 was selected for turther study and cloned into an RNAi vector, pANDA which was introduced into cell suspension cultures to knockdown the enzyme expression level. The knockdown effect was accessed via northern blotting and qRT-PCR analysis at the gene level and by LC-MS at the primary and secondary metabolite level. Quantitative RT-PCR revealed a significant reduction in C4H1 gene expression in the knockdown cell line compared to the wildtype control cell line. Data from northern blotting supported that the reduction of the gene expression resulted from aberrant small homologous small RNA production triggered by the dsRNA and followed by specific RNA degradation.

Reduction in secondary metabolite production in cell suspension cultures suggested that the enzyme C4H1 is crucial in biosynthesis of the compounds pinostobin and alpinetin, two compounds accessed in this study. Overexpressing this enzyme isomer could be carried out in the future to enhance the production of these compounds in *B. rotunda* cell suspension culture. Nevetheless, the availability of a *B. rotunda* genome sequence in the future would greatly ease the work of mining the suitable targets for metabolic engineering in order to enhance the compound production.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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Boon Chin Tan, Siew Kiat Tan, **Sher Ming Wong**, Nabeel Ata N., Noorsaadah Abdul Rahman, and Norzulaani Khalid. (2015). Distribution of flavonoids and cyclohexenyl chalcone derivatives in conventional propagated and in vitro-derived field-grown of *Boesenbergia rotunda* (L.) Mansf. *Evidence-Based Complementary and Medicine. Vol. 2015*. Article ID 451870. 7 pages. http://dx.doi.org/10.1155/2015/451870. (*ISI-Cited Publication*)

Hao Cheak Tan**, Sher Ming Wong**, Boon Chin Tan, and Norzulaani Khalid. (2015) A medicinal ginger, *Boesenbergia rotunda*: from cell suspension cultures to protoplast derived callus. *Sains Malaysiana* (accepted) (*ISI-Cited Publication*)

Noor Diyana Md. Mustafa, Norzulaani Khalid, Huan Gao, Zhiyu Peng, Mohd Firdaus Alimin, Noraini Bujang, **Sher Ming Wong**, Yusmin Mohd. Yusuf, Jennifer Ann Harikrishna, and Rofina Yasmin Othman. (2014).Transcriptome profiling shows gene regulation patterns in a flavonoid pathway in response to exogenous phenylalanine in *Boesenbergia rotunda* cell culture. *BMC Genomics 15*:984. DOI: 10.1186/1471-2164-15-984 (*ISI-Cited Publication*)

Sher Ming Wong, Nursafina Salim, Jennifer Ann Harikrishna, and Norzulaani Khalid (2013). Highly efficient plant regeneration via somatic embryogenesis from cell suspension cultures of *Boesenbergia rotunda*. *In Vitro Cellular & Developmental Biology - Plant 49(6)*: 665-673. (*ISI-Cited Publication*)

Eng Chong Tan, Saiful Anuar Karsani, Gen Teck Foo, **Sher Ming Wong**, Noorsaadah Abdul Rahman, Noorzulaani Khalid, Shatrah Othman, and Rohana Yusof. (2012). Proteomic analysis of cell suspension cultures of *Boesenbergia rotunda* induced by phenylalanine: identification of proteins involved in flavonoid and phenylpropanoid biosynthesis pathways. *Plant Cell Tissue and Organ Culture 111*: 219-229. (*ISI-Cited Publication*)

Eng Chong Tan, Yean Kee Lee, Chin Fei Chee, Choon Han Heh, **Sher Ming Wong**, Christina Li Ping Thio, Gen Teck Foo, Norzulaani Khalid, Noorsaadah Abd Rahman, Saiful Anuar Karsani, Shatrah Othman, Rozana Othman, and Rohana Yusof. (2012). *Boesenbergia rotunda*: from ethnomedicine to drug discovery. *Evidence-Based Complementary and Alternative Medicine Vol. 2012*. Article ID 473637. 25 pages. (*ISI-Cited Publication*)

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Norzulaani Khalid, Norazma Yusuf, Noor Diyana Md. Mustafa, **Sher Ming Wong**, and Eng Chong Tan. (2012). Manipulation of *In Vitro* cultures of *Boesenbergia rotunda* for enhanced production of targeted bioactive compounds in the phenylpropanoid biosynthesis pathway. In *4th Australasian Metabolomics Symposium*, 04 Sep 2012 to 05 Dec 2012, UiTM, Kuala Lumpur.

APPENDIX

Appendix A: SCV of fine, embryogenic cell suspension

Day	SCV 1	SCV 2	SCV 3	Means ± SE
0	0.5	0.5	0.5	0.5 ± 00
5	0.8	0.8	0.9	0.8 ± 0.02
10	1.2	1.2	1.5	1.3 ± 0.05
15	2.5	2.7	3	2.7 ± 0.08
20	3.5	3.5	3.8	3.6 ± 0.05

Appendix B: Statistical analysis on the number of SE regenerated on different inoculation SCV plated

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	concentration	Statistic	df	Sig.	Statistic	df	Sig.
VAR00002	1	.375	3	.	.775	3	.057
	2	.178	3	.	.999	3	.956
	3	.253	3	.	.964	3	.637
	4	.297	3	.	.917	3	.443

a. Lilliefors Significance Correction

Descriptive Statistics

Dependent Variable:VAR00002

concentration	Mean	Std. Deviation	N
1	2.8667E2	134.00498	3
2	88.6667	50.01333	3
3	1.4978E2	28.51380	3
4	1.0267E2	13.05118	3
Total	1.5694E2	102.84389	12

Tukey HSD

concentration	N	Subset 1	Subset 2
2	3	88.6667	
4	3	1.0267E2	1.0267E2
3	3	1.4978E2	1.4978E2
1	3		2.8667E2
Sig.		.742	.060

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 5360.509.

Appendix C: C4H gene sequences isolated

*Primers sequences highlighted in lines

>C4H1

GTCAAGTTCGGACAGTTACCAGTGGTGGCGTGCATTCCCTTCCTCTTCGCC
CTCCCGTTCTTTTTCGTGACTTATGGCGGCGGCGGTGGTAAAACTCCGCCTG
GCCCCGTCGCTTTGCCCATCTTCGGCAACTGGCTCCAGGTGGGGAATGACCT
CAACCACCGGAATCTGGTGGGGATGGCTAAGAAGTACGG**AGATGTGTTCC**
TGTTGCGGCTGGGCGTGCGGAACCTGGCGGTGGTCTCCGACCCCAAGCTCG
CCGCCGAGGTGCTCCACACGCAGGGCGTGGAGTTCGGCTCGCGGCCGCGCA
ACCTGGTGTGGGACATCTTCACCGACAGCGGCAAGGACATGGTGTTCACGG
AGTACGGCGACCACTGGCGCAAGATGCGCCGGATCATGACCATGCCCTTCT
TCACTAATAAGGTGGTGGTGCAGTATCGGGGGATGTGGGAGGAGGAGATGA
ACGCGGTGGTGGAGAACCTGCGGGCGGCGCCGGCGGAGGGCGTCGTGGTG
CGTCGCCGCCTCCAGCTCATGCTCTACAACATCATGTACAGGATGATGTTTG
ACGCGCGGTTCGAGTCGGCGGAGGACCCTCTGTTCCAGCAAGCTACGCGGT
TCAACTCGGAGCGGAGCCGCCTCGCGCAGAGCTTCGATTACAACTACGGCG
ACTTCATCCCCATCCTGAGGCCCT**TCTTGAAAGGCTACTTGGAG**AAATGCA
GGGACCTGCAGAGCCGCCGGCTCGCCTTCTTCAACGATAACTACGTCGAGA
AAAGAAGGAAGGTGATGTCCGCCAGAGACGGAAGCAGCGACCGGCTGAGG
TGCGCCATGGACTACATCCTCGAAGCAGAGATGAACGGAGAGATCAGCTCC
GATAACGTCATCTACATCGTTGAGAACATTAACGTTGCAGCCATAGAGACG
ACGTTGTGGGGAATGGAGTGGGCACTGGCGGAGCTGGTGAACCACCCGAGT
TGTCAGAAGCGGCTCCGAGAGGAGCTTCAGCGAGTCCTGGGCCGAGGAGCC
CGGTGACGGAGACAAGCCTGCCTCGGCTGCCGTACCTGCAGGCGGTGGTGA
AGGAGACGCTCCGGCTGCACTCGCCGATCCCGCTGCTGGTCCCCCACATGA

ACCTCGACGCGGCGAAGCTCGGTGGATACGAAATCCC**CAAGCGGACGAAG**

GTCATCGTGAACGCGTGGTGGCTGGGGAACAACCCGGAGTGGTGGGTGCG

GCCGGAGGAGTTCAGGCCGGAGAGGTTCCTGGAGGAGGAGGGGGAGGTGG

AGGCGGTGGTAGGCGGCGGGAAGGTGGACT

>C4H7

GGGCAGCTCGAATTGTGGACGAGACGGCCGAGGGAGATGCCAATGATGGG

CAGGGCGAGGACGATTCCGGGACAGCTTCGCCGGCCAACTCCGAAGGGCAG

GAAACGGAAGTCGTTGCCGTTGGCCTCCACGCCGGCCTCCTCCTGCAGGAA

CCGCTCCGGCCGGAACTGCTCGGGGTCCTTCCACAGGGCCGGGTTGTTGGCC

AGCCACCAGGCGTTGACCAGGATCTTGCTCTCGGCGGGGACGTCGAAGCCG

GCGAGCTTGGCGTCGTGCAGGTTCATGTGGGGGACCAGGAGCGGGATGGAC

ATCCGCAGCCGGAGGGTCTCCTTCACCACCGCGTTGAGGTAAGGGAGGCGG

ACGAGGTCAGGCTCCGTCAGCTGGGCCGACCCGAGGACGGCGTCTAGCTCT

CGCCGGAGCTTGTTCTGGATCGCCGGGTGGTTCACCAGCTCCGCGATACCCC

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GACGTAAATATATAAACTTCATGAGAAAATAAGAAAACAGAGGAAAAGTA

ATGACCGGCGACGTTGATGTTCTCCACGATGTAAAGGACATTGTCATAGTTG

ATCTCGCCTCTCCTCTCCGCGTCCAAGATGTGATCCATTGCGCATTTGAGCT

CAAATTTCGATCCCTCCTCCTCCATCATCTTCCTGTTTACGGAAATCTTTAGT

CCCCACAAAAAAACAGAGCGATCGAAGGCAGCAAGACACGCACTTCCTCTT

GGCGACGAATTGTTCGTGGAAGAGTCACAACCGCCGTTCGTTCACCTTCCTG

CACCTGTTGAGGTATCCCCTTCACCATGGGCATCGGCCCTGGGATGAGGTCG

CCGTAGTTAAACTCAAAGCTCTAAATCTGAAGGCTCCGCTCAAGTTGAACG

CCTTCACCTTGTTGAAAGGGGAAACGGCATCGCTCTCGAACCGCAAGCCGA

AGTTGAAACCGAAACATGTAGTTGTACTTGATTAACTTGAAGGGTGAGCGG

GAACCCAATCCCTTCGTGGCCGGGTTCCGGATTTGGCTTATTTCCTTCCCCCC

AAACCGAAATTCCTATTCCCCCCCCCCTTCCTGGTTCCTGTTNACCCCCGGA

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AANGGAAAGGGTTTGATTTTGGTATTAACCCTTTGCCGTGTGAAAAGA

>C4H9

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AGCGCGAGGACGATGCCGGGGCAGTTGCGGCGCCCGGCGCCGAAGGGCAG

GAAGCGGAAGTCGTTGCCGTTGGCCTCCAAGCCGGCCTCCTCCTCCAGGAA

CCGCTCCGGACGGTACTGGTCGGGGCCTTCCCGGGGGGGTTTTGCCCACCAC

CCGTGTGACAAGATCTGTCTCCGGGAATTTAAACCCCCGATTTGGTTTGTAC

AGTTCATGGGGGGCCCAGGAGGGGATGCCCTCCGCACCGGAGATTTTTTTC

ACCACGCTTTATGTAGGGAGGCGGACGAGTCGGTCTCCTAAAATGGGGGGA

ACCCAGGGGGGTGTCCAGTTCCCGCGAGAGCTTTCTCTAGATCCCCGGGGT

GTTCACCATCTCCGATATGCCACACTCGATCGACCACAGAGTGGTCTCTAAC

GCTGTATTTAGTTCAAATTCAACGCAATAAATACACACGTAAACTTGCCGAG

ATATGAGAAAACAAAGGAAAATCAATTACCCGGCGACGTTGATGTTCTCGA

CGATGTAGAGGACATTGTCGTAGTTGATCTCGCCTCTCCTCTCCGCATCCAA

GATATGATCCATTGCACATTTGAGTTCAAACTTCGATCCCTTCTCCTCCATCA

TCTTCCTGTGTTTAGCTCCCAAAATTAACCTCCGCTAAAAATTTGCTAAAAC

AGAGCGATCGAACAAACCAAACCACGCACTTTCTCTCAGATACGAAGTGCT

CGTGGAAGATTCGCAACCGGCGGTCCTTCACTTCCCTGCACTTGTTGAGGTA

GCCCCTCAAGAAGGGGCGGAGAACCGGAATGAAGTCTCCGTAGTTGAACTC

GAAGCTCTGCGACAGCCGGCTCCTCTCGAAGTTGATCGCCTTAAGCTTGTTG

AACAGGGGATCCTCCTGGCTCTCGAATCGCCTGTCGAACATGATCCTGAAC

ATGTTGTTGTACATCATCAGCTGGAGGCGTCGCCGGAGCACCACTCCATCGC

TGGCTGCCTTTGGGTTGCTCCTCAGCTCCTCCACCACCAGCCGTATCTCCTCT

TCCCATCCCTCTCTATTCTGCTGCACCACCTGTTCCCCGCACCAGAACAAAG

GTCCAATTTTTATACACGGAATCATCAAAAGGCAGGATTTTGAATCGATTTA

TACCTTGTTGGTGAAGAAGGGAACCGTCATGATGCGCCGCATCTTGCGCCA

GTGGTCGCCGTAGACGGTGAAGACCACTCATGGACTTGGCCGGTGAAGATA

TCGAGGAAATCGTGCAGTGTGCGGGACCGACCTAGACAAAAAAGTCGGCGC

TTCGAGTATAAAAAGGACATTAAAGGAGAAGATCAACCAGAGTGTGACGGT

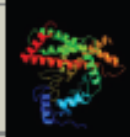
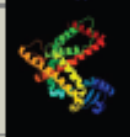
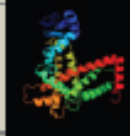
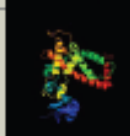
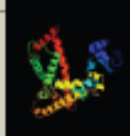
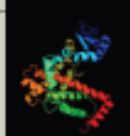
TCCC

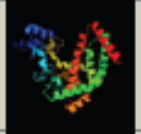
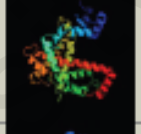
Appendix D: Phyre2 hit_report of C4H1 3-D modelling

Phyre2

Email	glaucous_83@yahoo.com
Description	C4H_expressed_in_e_coli
Date	Tue Jan 13 04:15:03 GMT 2015
Unique Job ID	1b9e357e00303a27

Detailed template information

#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c3e4eA_	Alignment		100.0	20	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 2e1; **PDBTitle:** human cytochrome p450 2e1 in complex with the inhibitor 4-2 methylpyrazole
2	d1r9oa_	Alignment		100.0	19	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
3	c3ns0B_	Alignment		100.0	14	**PDB header:**oxidoreductase, electron transport **Chain:** B: **PDB Molecule:**cholesterol side-chain cleavage enzyme, mitochondrial; **PDBTitle:** crystal structure of human cyp11a1 in complex with 20,22-2 dihydroxycholesterol
4	d2nnja1	Alignment		100.0	21	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
5	c2iagA_	Alignment		100.0	12	**PDB header:**isomerase **Chain:** A: **PDB Molecule:**prostacyclin synthase; **PDBTitle:** crystal structure of human prostacyclin synthase
6	d1po5a_	Alignment		100.0	21	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
7	d2j2a1	Alignment		100.0	18	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
8	c3jusA_	Alignment		100.0	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**lanosterol 14-alpha demethylase; **PDBTitle:** crystal structure of human lanosterol 14alpha-demethylase (cyp51)
9	c2x2n8_	Alignment		100.0	12	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**lanosterol 14-alpha-demethylase; **PDBTitle:** x-ray structure of cyp51 from trypanosoma brucei in complex2 with posaconazole in two different conformations
10	d1nr6a_	Alignment		100.0	21	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
11	c3ebsA_	Alignment		100.0	20	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 2a6; **PDBTitle:** human cytochrome p450 2a6 i208s/i300f/g301a/s369g in complex2 with phenacetin

12	d3cxha1	Alignment		100.0	17	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
13	c3hf2A_	Alignment		100.0	19	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**bifunctional p-450/nadph-p450 reductase; **PDBTitle:** crystal structure of the i401p mutant of cytochrome p450 bm3
14	c3k9v8_	Alignment		100.0	16	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**1,25-dihydroxyvitamin d(3) 24-hydroxylase, **PDBTitle:** crystal structure of rat mitochondrial p450 24a1 s57d in2 complex with chaps
15	c4fdhA_	Alignment		100.0	14	**PDB header:**oxidoreductase/oxidoreductase inhibitor **Chain:** A: **PDB Molecule:**cytochrome p450 11b2, mitochondrial; **PDBTitle:** structure of human aldosterone synthase, cyp11b2, in complex with2 fadrozole
16	c2rchA_	Alignment		100.0	10	**PDB header:**lyase **Chain:** A: **PDB Molecule:**cytochrome p450 74a; **PDBTitle:** crystal structure of arabidopsis thaliana allene oxide synthase (aos,2 cytochrome p450 74a, cyp74a) complexed with 13(s)-hod at 1.85 a3 resolution
17	c2hi4A_	Alignment		100.0	22	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 1a2; **PDBTitle:** crystal structure of human microsomal p450 1a2 in complex2 with alpha-naphthoflavone
18	c2ve3A_	Alignment		100.0	16	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative cytochrome p450 120; **PDBTitle:** retinoic acid bound cyanobacterial cyp120a1
19	c3sz1D_	Alignment		100.0	20	**PDB header:**oxidoreductase **Chain:** D: **PDB Molecule:**steroid 21-hydroxylase; **PDBTitle:** crystal structure of bovine steroid of 21-hydroxylase (p450c21)
20	d2ciba1	Alignment		100.0	14	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
21	c3g1qC_	Alignment	not modelled	100.0	12	**PDB header:**oxidoreductase **Chain:** C: **PDB Molecule:**sterol 14-alpha-demethylase; **PDBTitle:** crystal structure of sterol 14-alpha demethylase (cyp51)2 from trypanosoma brucei in ligand free state
22	c4dxlA_	Alignment	not modelled	100.0	15	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**lanosterol 14-alpha demethylase; **PDBTitle:** saccharomyces cerevisiae lanosterol 14-alpha demethylase with2 lanosterol bound
23	c3nxkD_	Alignment	not modelled	100.0	20	**PDB header:**oxidoreductase/oxidoreductase inhibitor **Chain:** D: **PDB Molecule:**steroid 17-alpha-hydroxylase/17,20 lyase; **PDBTitle:** human cytochrome p450 cyp17a1 in complex with abiraterone
24	c3davB_	Alignment	not modelled	100.0	13	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450 7a1; **PDBTitle:** crystal structure of human cyp7a1
25	c3danA_	Alignment	not modelled	100.0	12	**PDB header:**lyase **Chain:** A: **PDB Molecule:**cytochrome p450 74a2; **PDBTitle:** crystal structure of allene oxide synthase
26	d1bgna_	Alignment	not modelled	100.0	16	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
27	c3eqmA_	Alignment	not modelled	100.0	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 19a1; **PDBTitle:** crystal structure of human placental aromatase cytochrome p450 in2 complex with androstenedione
28	c2f9qA_	Alignment	not modelled	100.0	18	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 2d6; **PDBTitle:** crystal structure of human cytochrome p450 2d6
						PDB header:isomerase

29	c3b898_	Alignment	not modelled	100.0	18	**Chain:** B: **PDB Molecule:**prostaglandin I2 synthase; **PDBTitle:** crystal structure of zebrafish prostacyclin synthase (cytochrome p4502 8a1) in complex with substrate analog u51605
30	c3pm0A_	Alignment	not modelled	99.9	17	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 1b1; **PDBTitle:** structural characterization of the complex between alpha-2 naphthoflavone and human cytochrome p450 1b1 (cyp1b1)
31	c3wl3A_	Alignment	not modelled	99.9	20	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative cytochrome p450; **PDBTitle:** distinct monooxygenase and farnesene synthase active sites2 in cytochrome p450 170a1
32	c3ixd8_	Alignment	not modelled	99.9	10	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structure of camphor-bound cyp101d1
33	c4ll54A_	Alignment	not modelled	99.9	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**terminal olefin-forming fatty acid decarboxylase; **PDBTitle:** structure of cytochrome p450 olet, ligand-free
34	c4dzyA_	Alignment	not modelled	99.9	10	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structures of cyp101d2 y96a mutant
35	c4r21B_	Alignment	not modelled	99.9	22	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450 family 17 polypeptide 2; **PDBTitle:** zebra fish cytochrome p450 17a2 with progesterone
36	c2q9fA_	Alignment	not modelled	99.9	17	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 46a1; **PDBTitle:** crystal structure of human cytochrome p450 46a1 in complex with2 cholesterol-3-sulphate
37	d1n97a_	Alignment	not modelled	99.9	19	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
38	c4r1zB_	Alignment	not modelled	99.9	20	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cyp17a1 protein; **PDBTitle:** zebra fish cytochrome p450 17a1 with abiraterone
39	c2wm5A_	Alignment	not modelled	99.9	13	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative cytochrome p450 124; **PDBTitle:** x-ray structure of the substrate-free mycobacterium2 tuberculosis cytochrome p450 cyp124
40	c2zbxA_	Alignment	not modelled	99.9	16	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450-su1; **PDBTitle:** crystal structure of vitamin d hydroxylase cytochrome p4502 105a1 (wild type) with imidazole bound
41	d1re9a_	Alignment	not modelled	99.9	11	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
42	d1izma_	Alignment	not modelled	99.9	14	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
43	c2z36A_	Alignment	not modelled	99.9	16	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 type compactin 3",4"- **PDBTitle:** crystal structure of cytochrome p450 moxa from nonomuraea2 recticatena (cyp105)
44	c4l0wA_	Alignment	not modelled	99.9	11	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**p450 monooxygenase; **PDBTitle:** structure of p450sky (cyp163b3), a cytochrome p450 from sklamycin2 biosynthesis (heme-coordinated expression tag)
45	c3tiktA_	Alignment	not modelled	99.9	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structure of cyp108d1 from novosphingobium aromaticivorans2 dsm12444
46	c3dbgA_	Alignment	not modelled	99.9	20	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative cytochrome p450; **PDBTitle:** crystal structure of cytochrome p450 170a1 (cyp170a1) from2 streptomyces coelicolor
47	c3a4hA_	Alignment	not modelled	99.9	13	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**vitamin d hydroxylase; **PDBTitle:** structure of cytochrome p450 vdh from pseudonocardia autotrophica2 (orthorhombic crystal form)
48	c3wxsA_	Alignment	not modelled	99.9	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative monooxygenase; **PDBTitle:** crystal structure of cytochrome p450revi
49	c3nv6A_	Alignment	not modelled	99.9	8	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structure of camphor-bound cyp101d2
50	c2wbvA_	Alignment	not modelled	99.9	13	**PDB header:**electron transport **Chain:** A: **PDB Molecule:**cytochrome p450-like protein xpla; **PDBTitle:** cytochrome-p450 xpla heme domain p21
51	c3rwd4_	Alignment	not modelled	99.9	13	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 alkane hydroxylase 1 cyp153a7; **PDBTitle:** structure of p450pyr hydroxylase
52	c3lqyA_	Alignment	not modelled	99.9	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 cyp125; **PDBTitle:** crystal structure of mycobacterium tuberculosis cytochrome p4502 cyp125, p212121 crystal form
53	c3p1aA_	Alignment	not modelled	99.9	19	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**oxy protein; **PDBTitle:** structure of oxye (cyp165d3), a cytochrome p450 involved in2 teicoplanin biosynthesis
54	c2fr7A_	Alignment	not modelled	99.9	16	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative cytochrome p450; **PDBTitle:** crystal structure of cytochrome p450 cyp199a2
55	c3tyxC_	Alignment	not modelled	99.9	15	**PDB header:**oxidoreductase **Chain:** C: **PDB Molecule:**putative cytochrome p450; **PDBTitle:** crystal structure of cyp105n1 from streptomyces

56	d1cpta_	Alignment	not modelled	99.9	10	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
57	c2xbkA_	Alignment	not modelled	99.9	14	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**pimd protein; **PDBTitle:** x-ray structure of the substrate-bound cytochrome p450 pimd2 - a polyene macrolide antibiotic pimaricin epoxidase
58	c1t2hA_	Alignment	not modelled	99.9	12	**PDB header:**unknown function **Chain:** A: **PDB Molecule:**cp450cin; **PDBTitle:** crystal structure of cytochrome p450cin complexed with its2 substrate 1,8-cineole
59	c2y46B_	Alignment	not modelled	99.9	13	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cp-450-like protein; **PDBTitle:** structure of the mixed-function p450 mycg in complex with2 mycinamicin iv in c 2 2 21 space group
60	c3p3oA_	Alignment	not modelled	99.8	13	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structure of the cytochrome p450 monooxygenase aurh (ntermii)2 from streptomyces thioluteus
61	c2y9tD_	Alignment	not modelled	99.8	14	**PDB header:**oxidoreductase **Chain:** D: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structure of substrate free cytochrome p450 stap2 (cyp245a1)
62	c2dcrA_	Alignment	not modelled	99.8	14	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**putative cytochrome p450 142; **PDBTitle:** crystal structure of mycobacterium tuberculosis cyp142: a2 novel cholesterol oxidase
63	c2c6hB_	Alignment	not modelled	99.8	15	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450 monooxygenase; **PDBTitle:** crystal structure of yc-17-bound cytochrome p450 plic2 (cyp107l)
64	d1ordaa_	Alignment	not modelled	99.8	17	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
65	d1s1fa_	Alignment	not modelled	99.8	13	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
66	c2dkkA_	Alignment	not modelled	99.8	14	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450; **PDBTitle:** structure/function studies of cytochrome p450 158a1 from streptomyces2 coelicolor a3(2)
67	c4mm0B_	Alignment	not modelled	99.8	13	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cp450-like monooxygenase; **PDBTitle:** crystal structure analysis of the putative thioether synthase sgvp2 involved in the tailoring step of griseoviridin
68	d1o5da_	Alignment	not modelled	99.8	13	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
69	c4jbtB_	Alignment	not modelled	99.8	15	**PDB header:**oxidoreductase/substrate **Chain:** B: **PDB Molecule:**cytochrome p450 monooxygenase; **PDBTitle:** the 2.2 a crystal structure of cyp154c5 from nocardia farcinica in2 complex with androstenedione
70	c3e5bA_	Alignment	not modelled	99.8	14	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 (cytochrome p450 hydroxylase); **PDBTitle:** crystal structure of cyp105p1 wild-type 4-phenylimidazole complex
71	c3bulA_	Alignment	not modelled	99.8	12	**PDB header:**metal binding protein **Chain:** A: **PDB Molecule:**calo2; **PDBTitle:** crystal structure of calo2
72	c2uvnB_	Alignment	not modelled	99.8	9	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450 130; **PDBTitle:** crystal structure of econazole-bound cyp130 from2 mycobacterium tuberculosis
73	c4e2pA_	Alignment	not modelled	99.8	14	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 107b1 (p450cvilb1); **PDBTitle:** crystal structure of a post-tailoring hydroxylase (hmtn) involved in2 the himastatin biosynthesis
74	c3os3A_	Alignment	not modelled	99.8	17	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**oxy protein; **PDBTitle:** crystal structure of the orf6* (cyp165d3) monooxygenase involved in2 teicoplanin biosynthesis
75	c2ijnA_	Alignment	not modelled	99.8	13	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 113a1; **PDBTitle:** structure of cytochrome p450 eryk in complex with its2 natural substrate erd
76	c3nc7A_	Alignment	not modelled	99.8	10	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 cypc; **PDBTitle:** cyp134a1 2-phenylimidazole bound structure
77	c3mqxB_	Alignment	not modelled	99.8	15	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**putative p450 monooxygenase; **PDBTitle:** crystal structure of p450 oxyd that is involved in the biosynthesis of2 vancomycin-type antibiotics
78	d1n40a_	Alignment	not modelled	99.8	12	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
79	c3ejdD_	Alignment	not modelled	99.8	12	**PDB header:**oxidoreductase/lipid transport **Chain:** D: **PDB Molecule:**biotin biosynthesis cytochrome p450-like enzyme; **PDBTitle:** crystal structure of p450biol in complex with hexadec-9z-2 enoic acid ligated acyl carrier protein
80	d1jfka_	Alignment	not modelled	99.8	10	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
81	[illegible]	Alignment	not modelled	99.8	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**fatty acid alpha-hydroxylase;

81	c3aawmA_	Alignment	not modelled	99.8	12	**PDBTitle:** cytochrome p450sp alpha (cyp152b1) wild-type with palmitic acid
82	c4fl6B_	Alignment	not modelled	99.8	14	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**putative p450-like protein; **PDBTitle:** crystal structure of the cytochrome p450 enzyme txte
83	d1gwia_	Alignment	not modelled	99.8	14	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
84	d1cl0a1	Alignment	not modelled	99.8	13	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
85	c4ubnA_	Alignment	not modelled	99.8	18	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**pentalenic acid synthase; **PDBTitle:** the crystal structure of cytochrome p450 105d7 from streptomyces2 avermitilis in complex with diclofenac
86	d1jfba_	Alignment	not modelled	99.8	13	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
87	d1uesa_	Alignment	not modelled	99.7	12	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
88	c3r9cA_	Alignment	not modelled	99.7	15	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 164a2; **PDBTitle:** crystal structure of mycobacterium smegmatis cyp164a2 with econazole2 bound
89	c3ofuB_	Alignment	not modelled	99.7	13	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450; **PDBTitle:** crystal structure of cytochrome p450 cyp101c1
90	c3abbA_	Alignment	not modelled	99.7	14	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 hydroxylase; **PDBTitle:** crystal structure of cyp105d6
91	c4qqnA_	Alignment	not modelled	99.7	11	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450 superfamily protein; **PDBTitle:** crystal structure of hmtt involved in himastatin biosynthesis
92	c3wecA_	Alignment	not modelled	99.7	9	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**cytochrome p450; **PDBTitle:** structure of p450 raua (cyp105a1) complexed with a biosynthetic2 intermediate of aurachin re
93	d1lp7a_	Alignment	not modelled	99.6	14	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
94	d1uesa_	Alignment	not modelled	99.6	14	**Fold:**Cytochrome P450 **Superfamily:**Cytochrome P450 **Family:**Cytochrome P450
95	c4tvfA_	Alignment	not modelled	99.5	12	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**oxyb; **PDBTitle:** oxyb from actinoplanes teichomyceticus
96	c2rfrB_	Alignment	not modelled	99.5	15	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**cytochrome p450; **PDBTitle:** ligand bound (4-phenylimidazole) crystal structure of a2 cytochrome p450 from the thermoacidophilic archaeon3 picrophilus torridus
97	d2ffeia1	Alignment	not modelled	80.7	17	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
98	d1wexa_	Alignment	not modelled	66.9	14	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
99	c2adbA_	Alignment	not modelled	61.1	20	**PDB header:**rna binding protein/rna **Chain:** A: **PDB Molecule:**polypyrimidine tract-binding protein 1; **PDBTitle:** solution structure of polypyrimidine tract binding protein2 rbd2 complexed with cucucu rna
100	d1ubea2	Alignment	not modelled	56.5	17	**Fold:**Anti-LPS factor/recA domain **Superfamily:**RecA protein, C-terminal domain **Family:**RecA protein, C-terminal domain
101	d1mo6a2	Alignment	not modelled	55.8	21	**Fold:**Anti-LPS factor/recA domain **Superfamily:**RecA protein, C-terminal domain **Family:**RecA protein, C-terminal domain
102	c2dnq6_	Alignment	not modelled	54.8	12	**PDB header:**rna binding protein **Chain:** A: **PDB Molecule:**rna-binding protein 4b; **PDBTitle:** solution structure of rna binding domain 1 in rna-binding2 protein 30
103	d1s4da1	Alignment	not modelled	51.7	12	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
104	c3sdu8_	Alignment	not modelled	46.7	15	**PDB header:**rna binding protein **Chain:** B: **PDB Molecule:**non-pou domain-containing octamer-binding protein; **PDBTitle:** crystal structure of a paraspeckle-protein heterodimer, pspc1/nono
105	c3ns5B_	Alignment	not modelled	46.6	12	**PDB header:**translation **Chain:** B: **PDB Molecule:**eukaryotic translation initiation factor 3 subunit b; **PDBTitle:** crystal structure of the rna recognition motif of yeast eif3b residues2 76-161
106	d1h2tz_	Alignment	not modelled	37.6	15	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
107	d1ilna_	Alignment	not modelled	36.0	23	**Fold:**STAT-like **Superfamily:**CAPPD, an extracellular domain of amyloid beta A4 protein

						Family:CAPPD, an extracellular domain of amyloid beta A4 protein
108	d1qhha_	Alignment	not modelled	33.3	20	**Fold:**DNA damage-inducible protein Dini **Superfamily:**DNA damage-inducible protein Dini **Family:**DNA damage-inducible protein Dini
109	d2qhpa2	Alignment	not modelled	32.7	12	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
110	d1xp8a2	Alignment	not modelled	32.2	13	**Fold:**Anti-LPS factor/recA domain **Superfamily:**RecA protein, C-terminal domain **Family:**RecA protein, C-terminal domain
111	c2e5pA_	Alignment	not modelled	31.1	13	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**u6 snrna-specific terminal uridylyltransferase 1; **PDBTitle:** solution structure of rna binding domain in rna binding2 motif protein 21
112	c2dnoA_	Alignment	not modelled	31.0	10	**PDB header:**rna binding protein **Chain:** A: **PDB Molecule:**trinucleotide repeat containing 4 variant; **PDBTitle:** solution structure of rna binding domain in trinucleotide2 repeat containing 4 variant
113	c3s8sA_	Alignment	not modelled	30.7	18	**PDB header:**transcription **Chain:** A: **PDB Molecule:**histone-lysine n-methyltransferase setd1a; **PDBTitle:** crystal structure of the rrm domain of human setd1a
114	d1wbxa_	Alignment	not modelled	29.9	10	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
115	c2mkkA_	Alignment	not modelled	28.4	20	**PDB header:**translation regulator/rna **Chain:** A: **PDB Molecule:**cytoplasmic polyadenylation element-binding protein 1; **PDBTitle:** structural model of tandem rrm domains of cytoplasmic polyadenylation2 element binding protein 1 (cpeb1) in complex with rna
116	c4b3sA_	Alignment	not modelled	28.4	25	**PDB header:**ligase **Chain:** A: **PDB Molecule:**glutamine-trna ligase; **PDBTitle:** the structure of glutaminyl-trna synthetase from saccharomyces2 cerevisiae
117	d1nda_	Alignment	not modelled	27.7	22	**Fold:**Adenine nucleotide alpha hydrolase-like **Superfamily:**Nucleotidyl transferase **Family:**Class I aminoacyl-tRNA synthetases (RS), catalytic domain
118	d2cqca1	Alignment	not modelled	27.0	17	**Fold:**Ferredoxin-like **Superfamily:**RNA-binding domain, RBD **Family:**Canonical RBD
119	d1qtra2	Alignment	not modelled	26.7	25	**Fold:**Adenine nucleotide alpha hydrolase-like **Superfamily:**Nucleotidyl transferase **Family:**Class I aminoacyl-tRNA synthetases (RS), catalytic domain
120	c2jvnA_	Alignment	not modelled	26.5	10	**PDB header:**rna binding protein **Chain:** A: **PDB Molecule:**nucleolar protein 3; **PDBTitle:** segmental isotope labeling of npl3

Appendix E: Chemical and buffer reagent formulation

Homogenization buffer (1000 ml)

100 mM Tris-HCl – 15.76 g
20 mM EDTA – 40 ml of 0.5 M, pH 8.0 EDTA
2% w/v CTAB – 20 g
1.42 M NaCl – 81.8 g
2 % w/v PVP- 40 – 20 g
5 mM ascorbic acid – 0.88 g
4.0 mM DIECA – 0.96 g
*Bring volume to 1000 ml, autoclaved prior to use and kept at room temperature.

TE Buffer

pH 7.4	10 mM Tris-Cl (pH 7.4) 1mM EDTA (pH 8.0)
pH 7.6	10 mM Tris-Cl (pH 7.6) 1mM EDTA (pH 8.0)
pH 8.0	10 mM Tris-Cl (pH 7.8) 1mM EDTA (pH 8.0)

50 X TAE buffer (1000 ml)

Tris base – 121 g
Glacial acetic acid – 28.55 ml
0.5 M, pH 8.0 EDTA – 50 ml
*Top up to 500 ml with dH_2O
*Diluted into 0.5 X TAE buffer prior to electrophoresis.
(10 ml 50 X TAE buffer in 990 ml dH_2O)

6 X Loading dye

10 mM Tris-HCl (pH 7.6)
0.03% Bromophenol blue
0.03% Xylene cyanol FF
60% Glycerol
60 mM EDTA

Appendix F: Mean value of LC-MS primary metabolite profiles in RNAi cell line L8 and wild type cell suspension cultures

ID	20140325_cntrl	20140325_l8
GLY	5.093652234	3.189834737
HOMOSERINE	4138.202003	4383.663612
GLN	16549.39775	91955.78521
HIS	22703.43841	41166.05293
PUTRESINE	136.7087835	387.4323737
S-ADENOSYL METHIONINE	15.70534535	11.32913141
SPERMINE	85.59397594	102.9288009
ARG	63743.91144	129468.3562
ALA	256.6038373	448.188936
ASN	1058.748049	472.2931835
ASP	6234.849929	2666.02822
GABA	3016.922761	2537.827736
GLU	740.666794	906.0086712
CITRULLINE	2623.344466	5703.511198
PRO	141.427239	181.4012915
ORN	1137.150795	1088.236645
PHE	13.96809351	9.028854472
CYTOSINE	223.3095276	284.3295664
ADENINE	1783.520812	1865.875292
GUANINE	1673.926137	2357.172827
THYMINE	525.4130786	727.2736721
VAL	15774.19907	17112.41118
TYR	5196.743769	5471.687436
TRP	16616.66476	43437.26413
HYDROXYPROLINE	509.0023472	392.8994412
LEU/ILE	21705.03424	23001.78075
LYS	19835.85036	114872.3932
MET	26.50171317	51.26751384
SPERMIDINE	82.22218809	202.6532635
URACIL	273.203467	172.4141101
CAFFEIC ACID	33.13898094	197.1396901
CARNOSINE	62.00439329	100.5106462
ANTRANILATE	1207.4005	261.878785

APPENDIX F continued

CREATINE	8.866935454	12.1652031
MALIC ACID	1014934.531	1919004.563
LACTIC ACID	1109.188875	2073.664708
GLUCONIC ACID	67380.12829	119149.0994
2-OXOISOVALERIC ACID	87944.18108	141672.149
CIS-ACONITIC ACID	1048998.576	497468.56
CITRIC ACID	32648101.08	33533844.75
OXALOACETIC ACID	3426.926792	10935.042
SHIKIMIC ACID	183.6065	206.0512083
2-OXOGLUTARIC ACID	395.992625	214.2557917
ISOCITRIC ACID	511718.7007	518364.5054
GLYOXLIC ACID	357.3947083	688.0027917
GLYCOLIC ACID	30.44116667	45.96045833
OXALIC ACID	170.573	311.996
RIBOSE-5-PHOSPHATE	1.787624213	2.933017757
3-PHOSPHOGLYCERIC ACID	2.174436945	2.761055792
GLYCEROL-3-PHOSPHATE	4.618004199	5.308414703
ERYTHROSE-4-PHOSPHATE	0.027694482	0.025109666
GLUCOSE-1-PHOSPHATE/ GLUCOSE-6-PHOSPHATE	0.005194185	0.003193005
XYLULOSE-5-PHOSPHATE	0.696602233	0.868796827
RIBULOSE-5-PHOSPHATE	1.625440676	2.889285477
FRUCTOSE-6-PHOSPHATE	2.008978157	1.672338895
FRUCTOSE-1,6-PHOSPHATE	0.253930103	0.311501195
GLUTHATHIONE (RED)	1.690828796	0.880661133
GLUTHATHIONE (OX)	70.16777618	34.19722726
SHIKIMIC-3-PHOSPHATE	0.200159604	0.076502953
6-PHOSPHOGLUCONIC ACID	0.068532107	0.079388517

Appendix G: Statistical analysis of primary metabolite profiles in RNAi cell line L8 and wild type cell suspension cultures

ID	Fold change (L8/ Cntrl)	Log2	T-Test
GLY	0.63	-0.68	0.315126
HOMOSERINE	1.06	0.08	0.849513
GLN	5.56	2.47	0.01295
HIS	1.81	0.86	0.062212
ARG	2.03	1.02	0.024383
ALA	1.75	0.80	0.01858
ASN	0.45	-1.16	0.115145
ASP	0.43	-1.23	0.124352
GABA	0.84	-0.25	0.504237
GLU	1.22	0.29	0.017257
CITRULLINE	2.17	1.12	0.017746
PRO	1.28	0.36	0.0637
ORN	0.96	-0.06	0.930818
PHE	0.65	-0.63	0.248657
CYTOSINE	1.27	0.35	0.010158
ADENINE	1.05	0.07	0.925244
GUANINE	1.41	0.49	0.194848
THYMINE	1.38	0.47	0.205413
VAL	1.08	0.12	0.84003
TYR	1.05	0.07	0.911781
TRP	2.61	1.39	0.055203
HYDROXYPROLINE	0.77	-0.37	0.68387
LEU/ILE	1.06	0.08	0.908903
LYS	5.79	2.53	0.012735
MET	1.93	0.95	0.157159
URACIL	0.63	-0.66	0.159781
CARNOSINE	1.62	0.70	0.118881
PUTRESINE	2.83	1.50	0.006651
CAFFEIC ACID	5.95	2.57	0.052908
ANTRANILATE	0.22	-2.20	0.120871
CREATINE	1.37	0.46	0.024587
MALIC ACID	1.89	0.92	0.00446
LACTIC ACID	1.87	0.90	0.000466
GLUCONIC ACID	1.77	0.82	0.080016
2-OXOISOVALERIC ACID	1.61	0.69	0.009408
CIS-ACONITIC ACID	0.47	-1.08	0.368904
CITRIC ACID	1.03	0.04	0.908872
OXALOACETIC ACID	3.19	1.67	0.081683
SHIKIMIC ACID	1.12	0.17	0.671704
2-OXOGLUTARIC ACID	0.54	-0.89	0.03428
ISOCITRIC ACID	1.01	0.02	0.954619
GLYOXLIC ACID	1.93	0.94	0.00223

APPENDIX G continued

GLYCOLIC ACID	1.51	0.59	0.262258
OXALIC ACID	1.83	0.87	0.024379
SHIKIMIC-3-PHOSPHATE	0.38	-1.39	0.029429
6-PHOSPHOGLUCONIC ACID	1.16	0.21	0.555506
RIBOSE-5-PHOSPHATE	1.64	0.71	0.466813
3-PHOSPHOGLYCERIC ACID	1.27	0.34	0.650989
GLYCEROL-3-PHOSPHATE	1.15	0.20	0.597285
S-ADENOSYL METHIONINE	0.72	-0.47	0.356532
SPERMINE	1.20	0.27	0.285145
SPERMIDINE	2.46	1.30	0.356532
ERYTHROSE-4-PHOSPHATE	0.91	-0.14	0.317183
GLUCOSE-1-PHOSPHATE/ GLUCOSE-6-PHOSPHATE	0.61	-0.70	0.451813
XYLULOSE-5-PHOSPHATE	1.25	0.32	0.452966
RIBULOSE-5-PHOSPHATE	1.78	0.83	0.434189
FRUCTOSE-6-PHOSPHATE	0.83	-0.26	0.725837
FRUCTOSE-1,6-PHOSPHATE	1.23	0.29	0.431402
GLUTHATHIONE (RED)	0.52	-0.94	0.018353
GLUTHATHIONE (OX)	0.49	-1.04	0.08195

Appendix H: Histochemical staining reagents for GUS assessments

Histochemical Staining Reagent

Stock Solution	Working Concentration	Total Volume		
		5 ml	10 ml	25 ml
0.2 M $NaPO_4$	0.1 M	2.5 ml	5 ml	12.5 ml
0.1 M KFe^{3+}	0.5 mM	25 µl	50 µl	125 µl
0.1 M KFe^{2+}	0.5 mM	25 µl	50 µl	125 µl
0.5 M EDTA	10 mM	100 µl	200 µl	500 µl
0.5 % Triton	0.1 %	1 ml	2 ml	5 ml
Methanol	20 % (v/v)	1 ml	2 ml	5 ml
20 mg/ ml X-Gluc	1.0 mM	250 µl	500 µl	1250 µl
dH_2O	-	100 µl	200 µl	500 µl

FAA Fixing Solution 100 ml

Absolute EtOH – 45 ml
Glacial acetic acid – 5 ml
Formaldehyde – 5 ml
dH_2O – 45 ml

Appendix I: Plant tissue culture media formulation and preparation

Murashige and Skoog (1962) MS basal nutrients formulation

Components	Concentration in Media (mg/ L)	Stock solution concentration
Macronutrients		
$CaCl_2.2H_2O$	440	
KNO_3	1900	
KH_2PO_4	170	10 X
$MgSO_4.7H_2O$	370	
NH_4NO_3	1650	
Micronutrients		
$CoCl_2.6H_2O$	0.025	
$CuSO_4.5H_2O$	0.025	
H_3BO_4	6.2	
KI	0.83	100 X
$MnSO_4.4H_2O$	22.3	
$Na_2MoO_4.4H_2O$	0.25	
$ZnSO_4.7H_2O$	8.6	
Vitamins		
Glycine	2.0	
Nicotinic acid	0.5	100 X
Pyrodoxine-HCl	0.5	
Thiamine-HCl	0.1	
Myo-inositol	100	1 X
Iron		
$FeSO_4.7H_2O$	27.85	100 X
Na2EDTA	37.25	

*The pH of media was adjusted to 5.7 prior to autoclaving.

Sterilisation methods

a. By steam

All glassware, equipments and media were sterilised in an autoclave with 121 $^{\circ}$C, pressure of 1.2 kgf/ cm^2 for 20 minutes.

b. By fitration

Filter sterilisation method was used as alternative for sterilisation of heat-labile, temperature-sensitive components such as certain plant growth regulators and antibiotics. All of the components that would be damaged by steam sterilisation methods are filter sterilised by passing through a 0.22 µm nitrocellulose fliter (Millex ® - GV, Millipore).

Appendix J: Bacterial cultures media and preparation

Yeast Extract Broth (YEB) – 1 Litre

Nutrient broth – 14.0 g
Yeast extract – 1.0 g
Sucrose – 5.0 g
Magnesium Sulphate – 10mM

Yeast Extract (YE) Agar Plate – 1 Litre

Nutrient agar – 28.0 g
Yeast extract – 1.0 g
Sucrose – 5.0 g
Magnesium Sulphate – 10mM

The pH of the media was adjusted to 7.5 prior to autoclaving. Media were left to cool to ≈ 50 $^{\circ}$C before adding antibiotics.

Appendix K: Plasmid extraction chemicals

(i) Solution I (50 ml)

1.25 ml of 1 M Tris
1.0 ml of 0.5 M EDTA
450 g glucose
Top up to 50 ml with dH_2O

(ii) Solution II (500 µl)

10 µl of 10 N NaOH
50 µl of 10 % (w/v) SDS
440 µl of dH_2O

(iii) Solution III (250 ml)

150 ml of 5 M Potassium acetate
28.75 ml of glacial acetic acid
71.25 ml of dH_2O

Appendix L: Quantitative RT-PCR Results (qRT-PCR) analysis of C4H gene expression in RNAi cell line L8 and wild type cell suspension cultures

Well	Sample Name	Target Name	Reporter	Quencher	RQ	RQ Min	RQ Max	Ct Mean	Delta Delta Ct
A1	NTC	C4H	FAM	NFQ-MGB	- -	- -	- -	- -	- -
A2	Cntrl	C4H	FAM	NFQ-MGB	1	0.96	1.041	27.909	0
A3	L1	C4H	FAM	NFQ-MGB	0.651	0.591	0.717	30.52	0.619
A4	L8	C4H	FAM	NFQ-MGB	0.749	0.715	0.786	31.957	0.416
A5	L3	C4H	FAM	NFQ-MGB	0.571	0.487	0.669	31.442	0.809
A6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
B1	NTC	C4H	FAM	NFQ-MGB	- -	- -	- -	- -	- -
B2	Cntrl	C4H	FAM	NFQ-MGB	1	0.96	1.041	27.909	0
B3	L1	C4H	FAM	NFQ-MGB	0.651	0.591	0.717	30.52	0.619
B4	L8	C4H	FAM	NFQ-MGB	0.749	0.715	0.786	31.957	0.416
B5	L3	C4H	FAM	NFQ-MGB	0.571	0.487	0.669	31.442	0.809
B6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
C1	NTC	C4H	FAM	NFQ-MGB	- -	- -	- -	- -	- -
C2	Cntrl	C4H	FAM	NFQ-MGB	1	0.96	1.041	27.909	0
C3	L1	C4H	FAM	NFQ-MGB	0.651	0.591	0.717	30.52	0.619
C4	L8	C4H	FAM	NFQ-MGB	0.749	0.715	0.786	31.957	0.416
C5	L3	C4H	FAM	NFQ-MGB	0.571	0.487	0.669	31.442	0.809
C6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
D1	NTC	C4H	FAM	NFQ-MGB	- -	- -	- -	- -	- -
D2	Cntrl	C4H	FAM	NFQ-MGB	1	0.96	1.041	27.909	0
D3	L1	C4H	FAM	NFQ-MGB	0.651	0.591	0.717	30.52	0.619
D4	L8	C4H	FAM	NFQ-MGB	0.749	0.715	0.786	31.957	0.416
D5	L3	C4H	FAM	NFQ-MGB	0.571	0.487	0.669	31.442	0.809
D6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
E1	NTC	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
E2	Cntrl	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
E3	L1	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
E4	L8	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
E5	L3	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
E6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
F1	NTC	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -

Appendix L Continued

F2	Cntrl	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
F3	L1	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
F4	L8	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
F5	L3	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
F6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
G1	NTC	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
G2	Cntrl	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
G3	L1	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
G4	L8	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
G5	L3	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
G6	Blank	- -	- -	- -	- -	- -	- -	- -	- -
H1	NTC	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
H2	Cntrl	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
H3	L1	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
H4	L8	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
H5	L3	b-actin	FAM	NFQ-MGB	- -	- -	- -	- -	- -
H6	Blank	- -	- -	- -	- -	- -	- -	- -	- -

- - Undetermined

Analysis Type = Singleplex

Endogenous control = B-actin

Reference Sample = Cntrl

RQ Min/ Max Confidence Level = 95.0

Appendix M: Publication articles