CLONING AND Agrobacterium-MEDIATED TRANSFORMATION OF GLUTAMATE DECARBOXYLASE (GAD) RNAi CONSTRUCT IN EGGPLANT (Solanum melongena)

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by

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LEE ZE HONG

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LIST OF ABBREVATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
AMP	Adenosine monophosphate
A. tumefaciens	*Agrobacterium tumefaciens*
A. vitis	*Allorhizobium vitis*
BAP	6-Benzylaminopurine
BLAST	Basic Local Alignment Search Tool
CAT	Chloramphenicol acetyltransferase
cDNA	complementary DNA
CHS	Chalcone synthase
Cm^R	Chloramphenicol resisance gene
Cry	Crystal protein
cv.	Cultivar
DATC	Death of *Agrobacterium*-transformed cells
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsRNA	Double-stranded ribonucleic acid
dT	Deoxythymine
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FAOSTAT	Food and Agriculture Organization of the United Nations
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase

GAD	Glutamate decarboxylase
GDH	Glutamate dehydrogenase
GFP	Green fluorescence protein
GMC	Genetically modified crop
GOGAT	Glutamate synthase
GS	Glutamine synthetase
GUS	β-glucuronidase
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
hpt	Hygromycin phosphotransferase gene
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LB	Luria Bertani
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
MSG	Monosodium glutamate
MW	Molecular weight
NAA	Naphthaleneacetic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCBI	National Centre of Biotechnological Information
nptII	Neomycin phosphotransferase II gene
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PG	Polygalacturonase

R. rhizogenes	*Rhizobium rhizogenes*
R. rubi	*Rhizobium rubi*
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
S. melongena	*Solanum melongena*
S. sisymbriifolium	*Solanum sisymbriifolium*
S. torvum	*Solanum torvum*
siRNA	Short interfering RNA
SOC	Super Optimal broth with Catabolite repression
spp.	Several species
SpR	Spectinomycin resistance gene
SSADH	Succinic semialdehyde dehydrogenase
TBE	Tris-Borate-EDTA
TCA	Tricarboxylic acid
T-DNA	Transfer-DNA
TDZ	Thidiazuron
TE	Tris-EDTA
Ti	Tumour inducing
TM	Trademark
Tris-HCL	Tris-Hydrochloride
OD	Optical density

USDA	United States Department of Agriculture
var.	Variety
Vir	Virulence

LIST OF SYMBOLS

A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
α	Alpha
&	And
bp	Base pair
β	Beta
cm	Centimetre
˚C	Degree Celsius
γ	Gamma
g	Gram
g/L	Gram over litre
g/M	Gram over molar
kbp	Kilo base pair
kcal	Kilocalorie
kg	Kilogram
kV	Kilovolt
L	Litre
µg	Microgram
µL	Microlitre
µM	Micromolar
mg	Milligram
mg/L	Milligram over Litre
mL	Millilitre

mM	Millimolar
ms	Millisecond
M	Molar
ng	Nanogram
ng/µL	Nanogram over microlitre
-	Negative or without
N	Normal
%	Percent
±	Plus or minus
psi	Pounds per square inch
rpm	Revolutions per minute
xg	Times gravity
–	To or between
V	Volt
w/v	Weight over volume

PENGKLONAN DAN TRANSFORMASI PENGANTARA *Agrobacterium* KONSTRUK RNAi GEN GLUTAMATE DECARBOXYLASE (GAD) DALAM TUMBUHAN TERUNG (*Solanum melongena*)

ABSTRAK

Solanum melongena (tumbuhan terung) tergolong dalam famili Solanaceae dan berasal sama dengan tumbuhan tomato dan tumbuhan ubi kentang. Tumbuhan terung merupakan tanaman yang sangat penting di seluruh dunia dan dikaji untuk memahami nilai perubatan, nilai khasiat dan peranannya sebagai model penyelidikan. Kegunaannya dalam rawatan bronkitis, asma, arthritis dan kencing manis telah dibukti. Buah terung kaya dalam kandungan mineral dan amalan pemakanan terung banyak bermanfaat kepada manusia. Glutamate decarboxylase terlibat dalam kitaran glutamate dengan menukar glutamate kepada γ-amino butyric acid (GABA). Kajian ini bertujuan untuk menyahaktifkan gen *GAD* dengan menggunakan konstruk RNAi dan mengenal pasti pengaruhannya dalam kandungan glutamate dan asid amino dalam tumbuhan terung transgenik. RNA telah diekstrak daripada daun *in vitro* tumbuhan terung cv. Ungu Bulat sebelum penjanaan cDNA dan juga amplifikasi serpihan gen *GAD* dengan menggunakan polymerase chain reaction (PCR). Selepas penyisipan serpihan *att*B pada kedua-dua hujung serpihan gen *GAD*, pengklonan Gateway telah dijalankan untuk menyisipkan serpihan gen *GAD* ke dalam vektor penderma pDONR221. Penyisipan serpihan gen *GAD* telah dikenalpasti dan diklonkan ke dalam vektor destinasi pK8GWIWG-PG-B4. Selepas itu, vektor pengekpresan pEXPR-PG-RNAi-GAD207 yang membawa serpihan gen *GAD*

ditransformasi ke dalam sel *Agrobacterium tumefaciens* C58 dan dikenal pasti melalui analisis PCR. kotiledon tumbuhan terung cv. Bulat Putih ditransformasi dengan konstruk RNAi gen *GAD* dan diregenerasi dengan asid naphthalene asetik (NAA) untuk regenerasi kalus. Kalus transgenik dianalisis dengan PCR untuk mengenal pasti kewujudan transgen. Lima daripada sepuluh klon kalus transgenik membawa konstruk RNAi gen *GAD* dan hal ini membuktikan kejayaan penyisipan transgen. Selain itu, pengoptimuman protokol regenerasi untuk tumbuhan terung cv. Ungu Bulat and May 15 Ungu Hitam juga dijalankan dengan mengunakan pelbagai jenis cytokinin. Penyelidikan ini telah mengenal pasti bahawa kotiledon tumbuhan terung cv. Ungu Bulat bertindak balas terhadap medium Murashige and Skoog (MS) yang mengandungi 2 mg/L zeatin selepas transformasi dengan menggunakan *A. tumefaciens* LBA 4404. Selain itu, kotiledon tumbuhan terung cv. May 15 Ungu Hitam yang telah ditransformasi dengan mengunakan *A. tumefaciens* C58 jenis asal dapat diregenerasi dengan medium MS yang mengandungi 0.2 μM thidiazuron (TDZ) dan mencatatkan kadar regenerasi 91.3%.

CLONING AND *Agrobacterium*-MEDIATED TRANSFORMATION OF GLUTAMATE DECARBOXYLASE (GAD) RNAi CONSTRUCT IN EGGPLANT (*Solanum melongena*)

ABSTRACT

Solanum melongena commonly named as eggplant or brinjal belongs to the family of Solanaceae, sharing the same ancestor with tomato and potato. It is an economically important crop worldwide, being well studied for its medicinal properties, nutritional values and its role as an alternative model plant. It is proven to be useful in the treatment of various diseases such as bronchitis, asthma, arthritis and diabetes as well as rich in minerals which are beneficial to the human diet. The glutamate decarboxylase (GAD) is involved in the glutamate cycle by converting glutamate into γ-amino butyric acid (GABA). This study involves the generation of an RNAi construct to knockout the *GAD* gene with the aim to investigate its effects on the glutamate and amino acid content of transgenic eggplants. RNA was extracted from the *in vitro* leaves of eggplant cv. Ungu Bulat followed by the synthesis of cDNA and amplification of *GAD* gene fragment using polymerase chain reaction (PCR). After the addition of *att*B flanking sites onto the both ends of the *GAD* gene fragments, Gateway cloning was performed to insert the *GAD* gene fragment into the donor vector pDONR221. Successful insertion of the *GAD* gene fragment was confirmed and then cloned into the destination vector pK8GWIWG-PG-B4. The expression vector pEXPR-PG-RNAi-GAD207 containing the *GAD* gene fragment was then transformed into *Agrobacterium tumefaciens* C58 cells and confirmed through PCR analysis. Eggplant cotyledons of cv. Bulat Putih were transformed with

the *GAD* RNAi construct and subjected to regeneration with napthaleneacetic acid (NAA) for callus regeneration and transformed calli were subjected to PCR for the confirmation of transgene. Five out of ten clones of transgenic callus harboured the *GAD* RNAi construct indicating successful insertion of the transgene. In addition, optimization of regeneration protocols for eggplant cv. Ungu Bulat and May 15 Ungu Hitam were also performed with different types of cytokinins. The current investigation had identified that eggplant cotyledons of cv. Ungu Bulat responded to Murashige and Skoog (MS) medium supplemented with 2 mg/L zeatin after the transformation using *A. tumefaciens* LBA 4404. Besides that, cotyledons of eggplant cv. May 15 Ungu Hitam transformed with wild type *A. tumefaciens* C58 was able to regenerate on MS medium supplemented with 0.2 μM thidiazuron (TDZ) with a regeneration rate of 91.3%.

CHAPTER 1

Introduction

Solanum melongena, commonly known as the brinjal, aubergine or eggplant is a member of the family *Solanaceae* and it is also an economically important crop worldwide, especially in the Asian regions (Nunome et al., 2003). It is one of the three most important cultivated vegetable crops in *Solanaceae* besides potato and tomato (Wang et al., 2008). Over centuries, wild eggplants have been bred and cultivated to obtain commercial varieties which have more intense colour, higher production yield and fewer prickles (Muñoz-Falcón et al., 2009). Besides *S. melongena*, two other species from the same genus are also often referred as eggplant and cultivated widely in African regions (Schippers, 2000). They are the scarlet eggplant (*Solanum aethiopicum*) and gboma eggplant (*Solanum macrocarpon*). Along with other wild relatives of eggplant, these two species have been crossbred with cultivated eggplant to transfer the traits of interest into *S. melongena* for genetic improvement (Prohens et al., 2012).

Eggplant is an annual herbaceous plant. The plant is shrubby, upright, compact, well branched and grows from 0.6 to 1.2 meters. Its root is fibrous and lignified whereas its leaves are large, simple lobed, arranged alternately on stem and covered with hairs on underside. The flowers are hermaphrodite and in either white or purple colour (Chen & Li, 1997; Sidhu et al., 2005). The fruit is fleshy berry with pendent like appearance. Depending on the cultivar, the shape of the fruit varies from long cylindrical, ovoid or oblong whereas the colour varies from yellow, green, violet, white and striped (Ayaz et al., 2015).

Eggplant is a good source of nutrients for consumers. It is high in fibre content and consists of significant amount of vitamins such as vitamin A, B1 and B6 as well as minerals such as calcium, potassium, magnesium and phosphorus (Raigón et al., 2008; Okmen et al., 2009). Besides that, it is also proven to be effective in treatment of several diseases such as diabetes, bronchitis, asthma and arthritis (Magioli & Mansur, 2005). Its medicinal properties are highly contributed by the phytochemicals in its fruits such as phenolic compounds and anthocyanins which have high antioxidant activity (Mori et al., 2013). The most abundant phenolic compound isolated and identified in eggplant is chlorogenic acid (Paganga et al., 1999), whereas the major constituent of anthocyanins in eggplant fruit is nasunin (Kayamori & Igarashi, 1994). Although eggplant is rich in phytochemicals, minerals, vitamins and dietary fibre, its content of essential nutrients is rather low (Mori et al., 2013). Eggplant is one of the important food sources for vegetarians especially in a group of vegetarians which practices fruitarian diet and consumes only fruits (Venderley & Campbell, 2006). The approximated value of total protein content in different cultivars of eggplant is only about 1.1 g per 100 g of eggplant fruit (Ayaz et al., 2015). This amount is rather low and is not significantly sufficient to supply proteins for vegetarian diets as the eggplant fruit is commonly used to substitute meat. Due to the presence of the phenolic compounds and alkaloids, the eggplant fruits are bitter and may be unpleasant and not palatable to certain groups of people (Gajewski, et al, 2009). In order to enhance the protein levels and flavour of eggplant fruits, elevating the glutamate level in eggplant fruits using *Agrobacterium*-mediated transformation seems to be a promising strategy since glutamate serves as the precursors of various types of amino acids as well as induces umami flavour.

Glutamate is the precursor for amino acids and its metabolism is regulated by several enzymes which are glutamate dehydrogenase (GDH), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate decarboxylase (GAD), making it an intermediate between carbohydrate and amino acid metabolism (Cammaerts & Jacobs, 1985). Lightfoot et al. (1999) reported that the protein levels of tobacco and corn were increased after introducing *gdhA* gene from *Escherichia coli* into their genomes. Transformation study was also carried out using tomato and *gdhA* gene from *Aspergillus nidulans* which encodes the NADP-dependent GDH enzyme. Transgenic tomato from this study showed a one-fold increase of glutamate level and twice to thrice of amino acids level as compared to control fruit (Kisaka & Kida, 2003). Kisaka et al. (2006) also reported another alternative of glutamate and amino acids elevation through the suppression of the *GAD* gene using antisense suppression to downregulate the conversion of glutamate to γ-aminobutyric acid (GABA). These attempts by various studies explained the possibilities in the elevation of amino acids especially glutamate and likely increase of protein levels in various plants of the Solanaceae family. The current study aims to clone the *GAD* gene fragment in an RNAi construct through the Gateway cloning system to knockout the *GAD* gene for the purpose of glutamate elevation and subsequently protein elevation.

1.1 Objectives of research

The objectives of this study are:

1. To isolate Glutamate Decarboxylase (GAD) gene fragment from eggplant (*Solanum melongena*).

2. To clone *GAD* gene fragment into RNAi Gateway expression vector and transform into *Agrobacterium tumefaciens* C58

3. To optimize the regeneration protocol for potential transformed explants tissue.

4. To produce transgenic callus of eggplant through *Agrobacterium*-mediated transformation.

CHAPTER 2

Literature Review

2.1 Eggplant

2.1.1 History and economic value

The Solanaceae, also known as nightshades, is a cosmopolitan family to which the genus *Solanum* belongs. It comprises of approximately 90 genera and more than 2000 species which are widely distributed all over the tropical and temperate regions (D'Arcy, 1991; Collonnier et al., 2001a; Olmstead et al., 2008). *Solanum* is the largest genus within the Solanaceae family, with almost one third of the species from this family belongs to this genus. Many of them are economically important crops worldwide, such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), chilli and bell peppers (*Capsicum* spp.) and eggplant (*Solanum melongena*). Besides that, some of the solanaceous species are also cultivated for other purposes, such as tobaccos (*Nicotiana* spp.) and bittersweet (*Solanum dulcamara*) that are cultivated for their medicinal properties as well as petunias (*Petunia* spp.) which are used as ornamental or bedding plants (Edmonds & Chweya, 1997; Ando et al., 1999; Knapp, 2002; Weese & Bohs, 2007; Daunay et al., 2008).

Solanum melongena with the common name eggplant, brinjal or aubergine belongs to the family of Solanaceae and is one of the most essential vegetables worldwide. It originated from Northeast India, Laos, Myanmar, Vietnam, Northern Thailand and Southwest China. Wild eggplants still can be found in these regions

(Lester & Hasan, 1991). The earliest information of this old world species was found in the Sanskrit documents where the eggplant was recorded in about 2000 years ago, suggesting that domestication of wild eggplant was already being carried out in ancient India and Indo-burma regions (Daunay & Janick, 2007). Based on the description on eggplant in the documentations, it is believed that it gained high popularity due to its culinary and medicinal properties. The cultivation of eggplant in China has started as early as the fifth century and migrated to the European countries and Japan during seventh century and eighth century respectively and further carried to North America by European settlers (Edmonds & Chweya, 1997; Doganlar et al., 2002; Magioli & Mansur, 2005; Sifau et al., 2014).

Eggplant is cultivated widely in temperate, tropics and subtropics regions (Sihachakr et al., 1994; Daunay, 2008). Eggplant is particularly favoured in Asian countries, the places where its domestication and cultivation started millenia ago. In India, it is even crowned with the name of ‘King of Vegetables’ (Daunay & Janick, 2007). According to the data from Food and Agriculture Organization of the United Nations (FAOSTAT, 2016), eggplant had reached a worldwide production yield of 50.19 million tonnes and world area harvested of 1.87 million hectares in year 2014 with the steady growth of approximately 13.79% and 8.64% respectively from year 2010 (Figure 2.1). In terms of production share by regions, Asia accounted for 93.9% of world production of eggplant in 2014 (Figure 2.2). Among the Asian countries, China is the main producer of eggplant, harvesting 29.49 million tonnes in 2014, followed by India which produced 13.56 million tonnes of eggplant (Table 2.1).

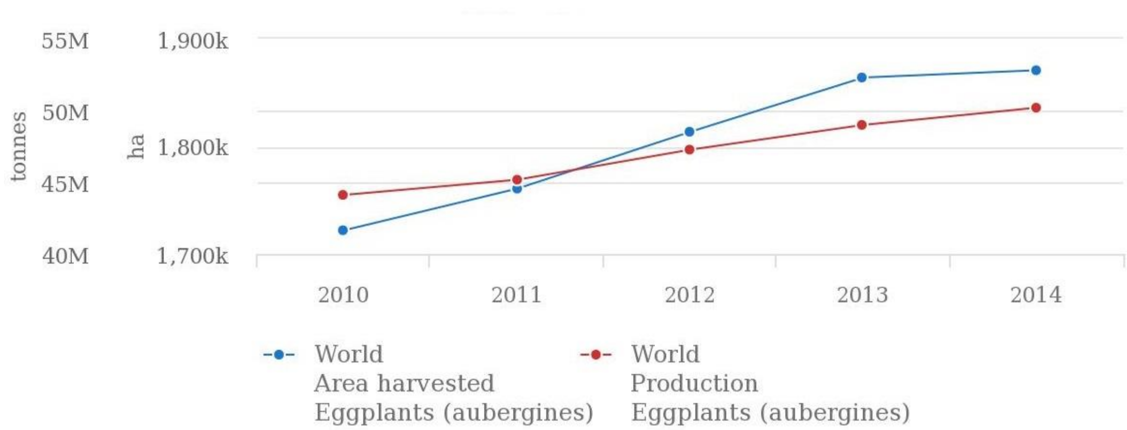

Figure 2.1: The production yield and area harvested of eggplants (aubergines) worldwide from year 2010 to 2014. Image adapted from FAOSTAT (2016).

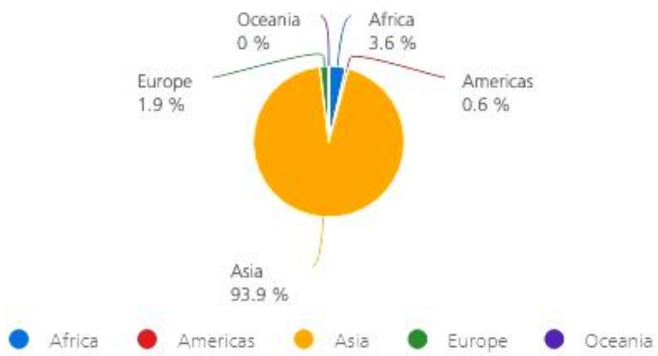

Figure 2.2: The production share of eggplants (aubergines) by regions in year 2014. Image adapted from FAOSTAT (2016).

Table 2.1: The top ten producers of eggplants (aubergines) in year 2014. Image adapted from FAOSTAT (2016).

Rank	Country	Production (tonnes)
1.	China	29,490,095
2.	India	13,557,820
3.	Egypt	1,257,913
4.	Turkey	827,380
5.	Iran	805,298
6.	Indonesia	557.053
7.	Iraq	434,322
8.	Japan	322,700
9.	Italy	308,722
10.	Phillipines	225,279

2.1.2 Botany and morphology

In general, there are three main varieties of eggplant, namely *S. melongena* var. *esculentum* (egg-shaped eggplant which includes white varieties and many cultivars of eggplant), *S. melongena* var. *serpentium* (long slender shaped eggplant, also known as snake eggplant) and *S. melongena* var. *depressum* (dwarf type) (Kashyap et al., 2003). Doganlar et al. (2002) and Frary et al. (2003) stated that wild primitive eggplants are prickly and produce small fruits with bitter taste. However, selection processes during the domestication resulted in the cultivars which have less prickles and produce larger and tastier fruits which are more suitable to be used as food. Besides the edibility of fruits, domestication of eggplant also caused genetic variation in different cultivars of eggplants all over the world in terms of yield, requirement for cultivation environment, fruit set as well as colour, size and chemical composition of fruits (Sekara et al., 2007).

Eggplant is a warm-weather crop that requires optimal temperature for growth and fruiting falls in the range of 21˚C to 29˚C whereas the high seed germination rate can be achieved with the average temperate range of 24˚C to 29˚C. It is sensitive to cold stress and growth of the young plants will be stunted if the night temperature is below 16˚C. Besides that, fruit set failure and pollen sterility can be induced by low temperature and light intensity. In terms of soil requirement, sandy loam or silt soils with the pH between 5.5 and 6.8 is highly recommended for eggplant growth (Chen & Li, 1996).

Eggplant is perennial in warm climate and it can grow into a bushy plant with the average height of 0.6 to 1.2 metre tall (Chen & Li, 1996), sometimes even reaching the height of three metres (Kowalska, 2008). According to Konys (1993), eggplant produces hermaphroditic flowers, with two to seven flowers arrange singly or in inflorescence. Flowers of eggplant are large in size (approximately three to five centimetres in diameter) with five to ten petals which are pink, white or purple in colour depending on the varieties and cultivars. There are 6 to 20 yellow coloured anthers arrange around the pistil (McGregor, 1976). Temperature and humidity of the surrounding environment are the key factors which affect the duration of pollination, pollen viability and occurrence of cross pollination (Prasad & Prakash, 1968; Swarup, 1995; Chen, 2001). Although eggplant is a self-pollinating crop, cross pollination still occurs occasionally between different eggplants to overcome the problem of partially self-incompatibility and also to induce better fruit set (Sekara & Bieniasz, 2008).

Eggplant produces multi-chamber fruits which are set singly or in cluster. Depending on the variety or cultivar, eggplant fruits can vary in shape, colour and size. Eggplant fruit is a fleshy berry with the pendent like appearance; the size varies from spherical to ovoid, oblong and long cylindrical shape (Figure 2.3) whereas the skin can be white, yellow, green, purple or black in colour (Naujeer, 2009). According to Frary et al. (2007), the diversity in fruit colour of eggplant is regulated by the presence or absence, combination and pattern of distribution of different types of chlorophyll and anthocyanin pigments. Fruit length of eggplant is in the range of 4 cm to 45 cm with the thickness between 3 cm and 35 cm in diameter and weight range of 0.5 g to 1.5 kg (Swarup, 1995).

Figure 2.3: **The different shapes and sizes of the eggplant fruit.**

2.1.3 Nutritional and medicinal values

2.1.3(a) Nutritional value

Eggplant is a popular vegetable which is commonly used in culinary and it had already appeared in the Western cuisines since the Medieval times (Adamson, 2004). It is popular for cooking and consumption because its fruit is high in nutritional values especially in vitamins and minerals contents, making it a good source of nutrients which is comparable to tomato (Som & Maity, 1986; Kalloo, 1993). In every 100 g edible portion of eggplant fruit, 92.3% of the content is water, while the rest of the nutritive content are carbohydrate (5.88 g), protein (0.98 g), lipid (0.18 g) and dietary fibre (3 g). Eggplant contains vitamin C, riboflavin, niacin, folic acid, vitamin B and vitamin K as well as minerals such as potassium, sodium, calcium, phosphorus and magnesium (Table 2.2).

The health benefits of eggplant are greatly contributed by the high fibre content and low carbohydrate level in its fruit. Hence, high consumption of eggplant fruits is effective in weight control and reducing the risk of cardiovascular disease. Besides that, eggplant fruit also contains various types of phytochemicals such as phenolic compounds (Hanson et al., 2006; Luthria & Mukhopadhyay, 2006) and anthocyanins (Sadilova et al., 2006; Azuma et al., 2008; Todaro et al., 2009) which had been proven to have significant effect in disease treatment such as diabetes and cancer (Hou, 2003; Katsube et al., 2003; Kwon et al., 2008). White eggplant fruit is higher in crude fibre content compared to cultivars which produce green or purple fruit. However, its anthocyanins and amino acids contents in fruits are much lower compared to those with coloured fruits (Lawande & Chavan, 1998).

Table 2.2: Nutritional value of eggplant per 100 g edible portion. Data adapted from National Nutrient Database for Standard Reference Release 28 slightly revised May, 2016 (USDA, 2016).

Nutrients	Value per 100 g edible portion
<u>Proximates</u>	
Water	92.3 g
Protein	0.98 g
Lipid	0.18 g
Total carbohydrate	5.88 g
Dietary Fibre	3 g
Energy	25 kcal
<u>Minerals</u>	
Calcium	9 mg
Iron	0.23 mg
Magnesium	14 mg
Phosphorus	24 mg
Potassium	229 mg
Sodium	2 mg
Zinc	0.16 mg
<u>Vitamins</u>	
Vitamin C	2.2 mg
Thiamin	0.039 mg
Riboflavin	0.037 mg
Niacin	0.649 mg
Vitamin B-6	0.084 mg
Folic acid	22 µg
Vitamin A	1 µg
Vitamin B-12	0.3 mg
Vitamin K	3.5 µg

2.1.3(b) Medicinal value

Eggplant fruit extract is capable of inducing inhibitory effect on α-amylase and α-glucosidase, thus reducing the risk of diabetes and diabetes-related diseases such as hypertension. Hence, eggplant has the potential for dietary management as well as prevention of diabetes and its relative diseases (Kwon et al., 2008; Nwanna et al., 2013). Nasunin is one the major anthocyanins which is present in eggplant fruits (Sakamura et al., 1963; Honda et al., 2012) whereas the most abundant phenolic acid compound in eggplant fruit extract is chlorogenic acid (Luthria & Mukhopadhyay, 2006). Nasunin is a free radical scavenger (Noda et al., 2000) and has been reported as one of the strongest antioxidants (Igarashi et al., 1993). Chlorogenic acid has been widely studied and it was reported to show high ability to prevent cancers, obesity and also cardiovascular diseases upon consumption (Tsuchiya et al., 1996; Hemmerle et al., 1997).

2.2 Breeding and sexual hybridization in eggplant

Pests and pathogenic attack are two of the main causes of massive damage to agricultural crops and subsequently incur enormous economic loss (Russell, 2013). For eggplant, most of the diseases are caused by microorganisms such as bacteria, fungi and viruses; whereas pests are mostly fruit and shoot borers as well as nematodes (Sihachakr et al., 1994; Srinivasan, 2009). Eggplant has low resistance against most of the pathogens and pests which cause wilting and reduced productivity in eggplant (Messiaen, 1989). In order to overcome this problem, eggplant has been hybridized with its wild relatives (Table 2.3) with higher resistance against the pathogens and wider genetic diversity to improve its quality and ensure better survival (Magioli & Mansur, 2005).

In conventional breeding of eggplant, intraspecific and interspecific crosses have been carried out to allow the introgression of important agronomical traits into the cultivated species (Collonnier et al., 2001a). However, this approach has been limited greatly by the sexual barriers between the species (Collonnier et al., 2001a) and the difficulties in obtaining fertile progenies after cross breeding (Gleddie et al., 1986b). Hence, in order to obtain successful hybrid from the interspecific cross, *in vitro* embryo rescue technique was performed by excising immature ovules or embryos from hybrids and culturing on medium (Bletsos et al., 1998). Although embryo rescue technique is effective to develop eggplant hybrids, the hybrids produced are either sterile or have extremely low pollen fertility (McCammon & Honma, 1983). As reviewed by Magioli & Mansur (2005), eggplant tissue shows a high morphogenetic potential and is suitable to be used as the model system in various fields of plant research such as developmental study, gene expression and regulation, genetic stability analysis for somaclones as well as establishment of biotechnological approaches for crops improvement. Therefore, other *in vitro* methodologies such as tissue culture approaches which include protoplast culture, somatic embryogenesis, organogenesis and somatic hybridization as well as genetic engineering have been introduced to eggplant and have achieved significant success since then (Magioli & Mansur, 2005).

Table 2.3: Sources of resistance to pathogens and pests in wild relatives of eggplant (*Solanum* spp.)

Pathogens and pests	Resistant wild species	References
Bacteria		
Ralstonia solanacearum	*S. americanum* Mill.	Daunay et al., 1991
	S. aethiopicum L.	Collonnier et al., 2001b
	S. torvum Sw.	Gousset et al., 2005
Fungi		
Fusarium oxysporum	*S. aethiopicum* L. gr. *gilo*	Rizza et al., 2002
	S. torvum Sw.	Gousset et al., 2005
Verticillium dahliae	*S. torvum* Sw.	Jadari et al., 1992
	S. abutiloides, S. scabrum & *S. toxicarium*	Iwamoto & Ezura, 2006
Phytophtora parasitica	*S. torvum* & *S. aethiopicum*	Gramazio et al., 2016
Insects		
Leucinodes orbonalis	*S. anomalum, S. gilo, S. incanum, S. indicum, S. integriifolium, S. khasianum, S. sisymbriifolium* & *S. xanthocarpum*	Srinivasan, 2008
Aphis gossypii	*S, mammosum* L.	Sambandam & Chelliah, 1983
Epilachna vigintioctopunctata	*S. viarum* Dun. & *S. torvum* Sw.	Sambandam et al., 1976
Tetranychus urticae	*S. macrocarpon*	Gisbert et al., 2006

Table 2.3 - Continued

Pathogens and pests	Resistant wild species	References
Tetranychus cinnabarinus	*S. sisymbrifolium* Lam. & *S. mammosum* L.	Schalk et al., 1975
Root-knot nematodes		
Meloidogyne spp.	*S. torvum* Sw.	Boiteux & Charchar, 1996
Viruses		
Potato virus Y	*S. linnaeanum* Hepper & Jegger	Horvath, 1984
Eggplant mosaic virus	*S. hispidum* Pers.	Rao, 1980
Others		
Mycoplasma (little leaf disease)	*S. hispidum* Pers.	Rao, 1980
	S. torvum Sw.	Datar & Ashtaputre, 1984

2.3 Plant tissue culture

2.3.1 Overview

Plant tissue culture is an aseptic culture system of plant cells, tissues and organs in a controlled environment under *in vitro* condition (Loyola-Vargas et al., 2008). In 1902, Gottlieb Haberlandt stated the possibilities of regenerating intact and functional plant from single cell and eventually proposed the theory of plant tissue culture (Krikorian & Berquam, 1969). The roles of plant hormones in plant tissue culture were rather unclear until the discovery of 6-furfurylaminopurine (kinetin) in 1955 as reviewed by Amasino (2005). Since then, different phytohormones which are present in plant cells naturally, namely absisic acid, auxins, cytokinins, ethylene and gibberellins as well as other synthetic chemicals (plant growth regulators) which produce hormone-like effects have been described with associated to their roles in plant growth regulation (Gaspar et al., 1996). Auxin and cytokinins promote cell morphological development and regulate organ differentiation in different manners and both of them are the main plant hormones which are usually involved in plant tissue culture work. However, formulating culture medium with optimal concentration and ratio of auxins and cytokinins is one of the greatest challenges for successful plant tissue culture since different plant species has different exogenous hormonal requirement, depending on the endogenous levels of phytohormones in the plant cells (Bhojwani & Razdan, 1986). Since eggplant is one of the important vegetable crops and it is emerging as the plant model system for the studies of important agronomical traits, tissue culture has been applied greatly to the regeneration of eggplant which serves as the effective tool for plant genetic engineering (Magioli & Mansur, 2005).

2.3.2 Tissue culture system in eggplant

2.3.2(a) Organogenesis and somatic embryogenesis

Organogenesis and somatic embryogenesis are the two alternative mechanisms which can be used to induce regeneration of plant other than utilizing protoplast culture (Jiménez, 2001). There are two different pathways of organogenesis: direct organogenesis from cultured explants such as leaves, stems, tubers rhizoids and other organs and also indirect organogenesis through callus to obtain shoot or root culture (Bridgen et al., 1994). The initial description of regeneration of eggplant species, *S. sisymbriifolium* Lam. based on indirect organogenesis of stem parenchyma cells was reported by Fassuliotis (1975). Direct and indirect organogenesis of *S. melongena* with different sources of explants such as hypocotyls (Muktadir et al., 2016), cotyledons (Bhat et al., 2013), stem nodes (Bhat et al., 2013), leaves (Bhatti et al., 2014) and roots (Ray et al., 2011) were also reported and recognized as the useful systems for eggplant regeneration.

Totipotency, is the ability of the undifferentiated cells to produce developmentally and morphologically normal embryo is the basis of somatic embryogenesis in plants (Komamine et al., 1992; Zimmerman, 1993). Since the first successful attempt of somatic embryos induction from carrot callus cells reported by Steward et al. (1958), somatic embryogenesis has been employed extensively to produce transgenic plants and artificial seeds as well as to study plant embryogenesis. Somatic embryo was first induced in eggplant by Yamada et al. (1967), using callus cultured with MS medium (Murashige & Skoog, 1962) supplemented with indole-3-

acetic acid (IAA), followed by successful attempts reported by other research groups. Matsuoka & Hinata (1979) reported the successful induction of somatic embryos from hypocotyls explants with high level (8.0 μg/mL) of napthaleneacetic acid (NAA); whereas Saito & Nishimura (1994) reported the use of another auxin which is 2,4-dichlorophenoxyacetic acid (2,4-D) is able to induce somatic embryogenesis in leaf and cotyledon explants.

2.3.2(b) Protoplast culture and somatic hybridization

A well established regeneration system via *in vitro* culture is essential for the application of further biotechnological techniques for plant improvement such as *Agrobacterium*-mediated transformation. Plant regeneration from eggplant protoplast culture has been studied widely since 1980s and successful attempts were reported in both *S. melongena* cultivars as well as its wild relatives such as *S. torvum* Sv. and *S. sisymbriifolium* Lam. (Bhatt & Fassuliotis, 1981; Jia & Potrykus, 1981; Gleddiei et al., 1985; Guri et al., 1987). Besides manipulating the concentration of different types of plant growth regulator (auxin and cytokinin), the genotype of the explants is also one of the important factors that affect the regeneration rate of protoplast culture (Gleddie et al., 1983).

Since the conventional method of sexual hybridization was found to be associated with the problems of sexual incompatibility and low hybrids fertility, somatic hybridization initiated from protoplast culture had become an alternative approach for crops improvement (Dudits et al., 1987; Glimelius et al., 1991). With the application of protoplast fusion technology, the transfer of important nuclear and