A REVIEW ON *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOLS OF TOMATO (*Solanum lycopersicum*)

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Final Year Project Report Submitted in
Partial Fulfilment of the Requirements for the
Degree of Bachelor of Science (Hons.) Technology and Plantation Management
in the Faculty of Plantation and Agrotechnology
Universiti Teknologi MARA

JULY 2019

DECLARATION

This Final Year Project is a partial fulfilment of the requirements for a degree of Bachelor of Science (Hons.) Technology and Plantation Management, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA.

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ACKNOWLEDGEMENTS

It is my honor to engrave this acknowledgement to convey my gratefulness to persons who have helped me through this paper review.

First of all, I would like to thank The Almighty God for successfully accomplish my thesis work. I offer my heartiest gratitude and special thanks to my supervisor Dr Yusuf Chong Yu Lok for his inspiring advice in each and every step of this study with affectionate guidance, valuable suggestions, soft behavior and for giving me the opportunity to work under her supervision to accomplish the dissertation.

I would like to thank intensely from my heart to my helpful seniors, batchmates and friends who provided immense encouragement, proper guidance, sound advice, good company, for their enthusiastic inspiration, corporation and sharing lots of good ideas throughout my thesis work.

I owe utmost gratefulness to my parents Rafi'ah Binti Ahmad and Mohamed Bin Sarno for their continuous support in every stage of my life. They have always supported me in my studies, provided me with the best possible academic environment, guided me with words of encouragement and made me confident to reach my aim. Finally, earnest thanks to my family members for their endless support to complete my thesis successfully.

AFIQ RIDZUAN BIN MOHAMED

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LIST OF ABBREVIATIONS

A.	*Agrobacterium*
ATP	Adenosine triphosphate
BAP	Benzylaminopurine
Cv	Cultivar
DNA	Deoxyribose Nucleic Acid
GFP	Green Fluorescent Protein
GUS	*β-glucuronidase*
LED	Light Emitting Diode
MS	Murashige and Skoog
mM	milli Mole
mm	milli meter
mL	milli Liter
NAA	Naphthaleneacetic acid
OD	Optical Density
T-DNA	Transfer DNA
T-region	Transfer region
Ti plasmid	Tumor inducing plasmid
UiTM	Universiti Teknologi MARA
UV	Ultra Violet
Vir	Virulence
YFP	Yellow Fluorescent Protein
%	Percentage

ABSTRACT

A REVIEW ON *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOLS OF TOMATO

(*Solanum lycopersicum*)

Tomato (*Solanum lycopersicum* L.) is one of the important vegetable crops around the world and also a suitable model plant for genetic study. Efficient transformation of tomato plant is important in order to get thorough understanding on a gene through functional study. However, the efficiency of genetic transformation of a plant depends on many factors. Hence, the purpose of this study is to assess and compare different transformation protocols of tomato. The transformation frequency of transient and stable transformation has been studied. To produce stable transformant, the transformation of tomato can be achieved through tissue culture-based and *in planta* transformation methods. There are several factors affecting the efficiency of tomato transformation including cultivar, explant, *Agrobacterium* strains, binary vector, temperature, co-cultivation period, pre-culture, incubation period, reporter gene expression and antibiotic concentration used of transformed tissue. All of these factors will determine the success of genetic transformation in tomato. The transformation of tomato cotyledon and stem explants was successfully performed through tissue culture-based transformation of *Agrobacterium tumefaciens*. This review revealed that *in planta* transformation method is more efficient compared to tissue culture-based method.

Keywords: tomato, transformation, factor, efficiency, Agrobacterium

ABSTRAK

ULASAN TENTANG PROTOKOL TRANSFORMASI AGROBAKTERIA SEBAGAI PENGANTARAAN DALAM TOMATO

(*Solanum lycopersicum*)

Tomato adalah salah satu tanaman yang penting di seluruh dunia dan juga model tanaman yang paling sesuai untuk kajian genetik. Transformasi yang berkesan pada tanaman tomato adalah penting bagi memastikan mendapat pemahaman yang mendalam pada genetik menerusi fungsi kajian. Tetapi, keberkesanan transformasi genetik pada tanaman bergantung pada banyak faktor. Oleh itu, tujuan utama kajian ini adalah untuk menilai dan membandingkan protokol transformasi yang berbeza pada tomato. Kekerapan transformasi sementara dan stabil telah dikaji. Bagi menghasilkan transformasi stabil, tranformasi tomato boleh dicapai menerusi tisu kultur dan cara transformasi dalam tumbuhan. Terdapat beberapa faktor yang yang mempengaruhi keberkesanan transformasi tomato termasuk genotip, jenis explant, strain Agrobakteria, vektor bina, suhu, tempoh penanaman, pra-kultur, tempoh inkubasi, ekspresi gen pelapor dan kepekatan antibiotik digunakan tisu yand diubah. Semua faktor ini akan menentukan kejayaan transformasi genetik pada tomato. Transformasi tomato untuk explant kotiledon dan batang telah berjaya dilakukan menerusi tranformasi pengantaraan agrobakteria tisu kultur. Ulasan ini mendedahkan cara transformasi dalam tumbuhan lebih berkesan berbanding cara kultur tisu.

Kata kunci: tomato, transformasi, faktor, keberkesanan, Agrobacteria

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Tomato (*Solanum lycopersicum* L.) originated from South America and its cultivation happened in Central America (Kimura and Sinha, 2008). Today, tomato has been an economically essential crop with huge production globally as the fruits contain many nutrients that beneficial to human health (Shikata and Ezura, 2016). Thus, tomato can be defined as one of the essential vegetable crops around the world. Breeding of tomato has been performed to improve the plant in term of productivity and fruit quality and also the ability of tomato to overcome abiotic and biotic stresses (Kimura and Sinha, 2008). Furthermore, several research which related to fruit such as ripening, salt tolerance and secondary metabolite have been done on tomato as it features suitable for scientific research (Shikata and Ezura, 2016). According to Kimura and Sinha (2008), fleshy fruit and compound leaves are the interesting features that the tomato plant has compared to other model plants. Fast life cycle and small size features which important for a suitable biological protocol make the tomato possible to combine the direct benefits of studying a crop species (Campos et al., 2010). In addition, Dan et al. (2007) stated that the tomatoes have good characteristics as a model system including small size, short life cycle which is 70-90 days from sowing to fruit ripening, small genome and transformability. The expression of foreign genes in plant tissues is an important key in plant biotechnology. The first transformation protocol for tomato was reported by McCormick et al., (1986) but the

transformation had resulted in low transformation efficiency. Hence, several approaches have been applied to produce transgenic tomato plant including tissue culture-based transformation and *in planta* transformation protocols in order to improve the transformation efficiency. In this review, the different transformation protocols of tomato plant were reviewed.

1.2 Problem Statement

Today, modern agriculture facing a major problem in order to produce a transgenic plant that resists to pest and disease as there is a huge application of a different chemical to control pest and disease which not eco-friendly (Solliman et al., 2016). This because currently plant transformation still used the protocols that tedious, time-consuming and costly and can sometimes lead to undesirable changes of plant characteristics (Yasmeen et al., 2008). Example, tissue culture which is one of the protocols in plant transformation was not an easy and rapid practice to produce a transgenic plant as it requires a large number of explants (Hasan et al., 2008). Hence, there is difficulty facing by the researcher in order to get the desirable result from plant transformation and produce transgenic plant in large scale and also hard to study gene expression in plant. In addition, according to Datta (2015), tissue culture-based transformation using cotyledons explant is time-consuming and requires a large number of explants but it showed low transformation frequency. In contrast, *in planta* transformation method can produce transformed seedling in short time and required a small number of explants as it showed higher transformation efficiency.

1.3 Objectives of Study

This review was conducted with the following objectives:

1. To analyze the available protocols of genetic transformation of tomato.
2. To recognize the easiest protocol to produce transgenic tomato.
3. To determine the most efficient transformation protocol for tomato.

1.4 Significance of Study

This study will help people from a different background such as students, researchers and farmers that use science knowledge related to the genetic engineering in order to meet their objectives or needs to produce a transgenic plant. It is because this study will provide them with vital information on how to produce a new transgenic plant in the correct manner from various protocols that had been discovered before. In addition, the production of the transgenic crop will help to overcome the pest and disease problem. Example the production of a transgenic crop of *Bacillus thuringiensis* maize will help farmers to fight against pest and disease that attack that corn thus reduce the negative impact on the amount of corn yield. Same goes to this study of tomato where the production of transgenic tomato may help to minimize the impact of the attack of pest and disease and resulting in high yield of tomato. In conclusion, this study will assist individuals learn and comprehend the right method of genetic engineering to create a transgenic plant and distinguish between each method.

CHAPTER 2

LITERATURE REVIEW

2.1 Global Tomato Production

Global tomato production is anticipated to boost for both fresh and processed tomatoes as the worldwide annual production of tomatoes has increased to about 300 percent over the last 4 centuries (Costa and Heuvelink, 2007). This occurs because the tomato is one of the extremely consumed vegetables in the globe, either as fresh or as a processing tomato where it can have many nutrient advantages as it is a source of vitamin A, vitamin C and vitamin B complexes such as thiamin, riboflavin and niacin (Nicola and Tibaldi, 2009). In addition, the top 10 major nations producing tomatoes are China, the USA, Turkey, India, Egypt, Italy, Iran, Spain, Brazil and Mexico where China has the largest cultivation area of 1,405,103 ha and an output of 32,5 tonnes (Nicola and Tibaldi, 2009). Normally, fresh tomatoes generated were sold on the open market while tomatoes generated for processing are manufactured under an agreement between growers and processors (Lucier, 2006).

According to Costa and Heuvelink (2007), the competition of greenhouse fresh tomato production will increase as both European country Spain and Netherlands will continue to improve greenhouse technology and aiming more sustainable production. Clearly, tomatoes require a relatively cool and dry climate for high yield and premium quality. However, the tomato can adapt to a broad spectrum of climatic circumstances from temperate to warm and humid tropical through distinct tomato varieties. (Naika et al.,

2005). Despite the fact that tomato is a perennial plant, it is cultivated as a hot seasonal annual crop owing to its sensitivity to frost where the tomato can be cultivated in open areas such as field planting or in close surroundings such as greenhouse when outdoor circumstances are not beneficial to the development of tomatoes (Nicola and Tibaldi, 2009). In addition, tomato plants required different temperatures at different growth stages (Table 2.1) which are seed germination, seedling growth, fruit set and red color development (Naika et al., 2005).

Table 2.1: Temperature requirements at different growth stages of tomato.

(Source: Naika et al., 2005)

Temperature (^oC)	**Growth Stages**			
	Seed germination	Seedling growth	Fruit set	Red color development
Minimum	11	18	18	10
Optimum range	16 - 29	21 - 24	20 - 24	20 – 24
Maximum	34	32	30	30

2.1.1 Tomatoes Production in Malaysia

In Malaysia, tomato can be classified as one of the important vegetables after pepper as its production is high with an average of 103.2 metric tons per hectare in 2013 (Rahim et al., 2017). The demands for healthy and safety tomatoes have been escalated in both export as well as domestic markets in Malaysia. The three states in Malaysia that have the largest planting site in order to produce highland variety tomato which is Pahang, Kelantan and Sabah with 2500, 198 and 110 hectares respectively (Ahmad et al., 2017). The most famous place for tomato cultivation and production in Malaysia is Cameron Highland

which located in Pahang State of Peninsular Malaysia and this is due to the cool temperature along year which makes Cameron Highland area a suitable place to cultivate good qualities tomatoes throughout the year (Ahmad et al., 2017). Other tomato planting areas are in Sarawak, Selangor, Johor and Melaka where these locations provide a conducive environment and temperature for cultivating tomatoes (Rahim et al., 2017).

Tomatoes with round, oblong and angular shape are the most popular cultivar of tomato varieties in Malaysia. For the highland tomato, the variety is the local type round tomato L24 which commonly planted in Cameron highland other than few other varieties and for the lowland soil, the famous commercial varieties are MT1, F1 and Serdang 2 where MT1 varieties were produced by the Malaysian Agricultural Research and Development Institution (MARDI) (Rahim et al., 2017). Furthermore, there are several ways to planting tomatoes which are conventional (open system), hydroponics (closed system) and fertigation (drip system) where the production of tomato in Malaysia can approximately double through modern irrigation system and the improvement on nutrient availability provided (Rahim et al., 2017). This irrigation system is considered as one of the important elements causing the increase in tomato yield. Hence, high production of tomato can meet the demand of the consumer either domestic or international (Ahmad et al., 2017).

2.1.2 The Health Benefits of Tomato

Tomato has been a popular fruit around the world not only because of its tastes, color and good texture but also because of the nutrients it contains. Thus, we can say that the tomato fruit is beneficial for human health. The tomato fruit is rich in vitamin C, A, B2, K and folate and it also has no trace of cholesterol and only a few amounts of lipid and calories of 18 kcal (Rahman, 2017). Hence, these perfect contents of tomato make it a perfect diet and a nutritional choice for adult, children and patients with a disease especially to whom have diabetes and heart disease (Kobylecki, 2015). Rahman (2017) mentioned that there is carotenoid found in tomato apart from those vitamins which are lycopene that also can deliver health benefits to whom consume it. Lycopene can help in reducing cell damage and it also has been proven to reduce the possibility of stroke in men (Karppi et al., 2012). In addition, Tan et al., (2010) stated that tomato also works against prostate cancer in men.

2.2 Tomato as a Model Plant

Model plant system plays a crucial role in order to understand plant biology. Before this Arabidopsis is the most popular model plant system due to its special features which are small size, rapid life cycle, small genome and transformability. But now, tomato can also be a successful model plant system as it is sharing some successful features with Arabidopsis (Dan et al., 2007). Any difficulty or impossibility research on Arabidopsis such as the formation of fleshy climacteric fruits and photoperiod-independent sympodial flowering can be done on tomato (Campos et al., 2010). In addition, tomato can be easily grown in a greenhouse with daylights between 8 and 16 hours, temperature between 10 to

35ºC and relative humidity between 30 – 90% (Schwarz et al., 2014). Tomato can be a convenient model plant system for researchers through appropriate cultivation techniques such as applying the optimum amount of fertilizer to ensure sufficient nutrient on tomato (Schwarz et al.,2014).

2.2.1 Micro-Tom Tomato (MT)

Micro-Tom is a small size tomato cultivar that was initially grown for the home gardening purpose (Scott and Harbaugh, 1989). Micro-Tom tomato shares some features with Arabidopsis such as short life cycle and small genome which make it an ideal model plant system for genetic transformation (Dan et al., 2007). Due to Micro-Tom successful features such as small genome and transformability, numerous protocols have been developed on it such as plant transformation, expressed sequence tags, the biological process of abiotic and biotic stress and others (Rothan et al., 2016). Currently, Micro-Tom has undergone tremendous progress in genetic transformation in order to study the functional genomics and economic trait development in crops (Dan et al., 2007). Micro-Tom cultivar has a great advantage in being able to grow up to 1357 plants/m^2 at different plant densities based on trays or pots used where high densities are used for genomic screening and low densities are used for physiological and molecular analysis (Rothan et al., 2016).

2.3 Genetic Transformation of Tomato

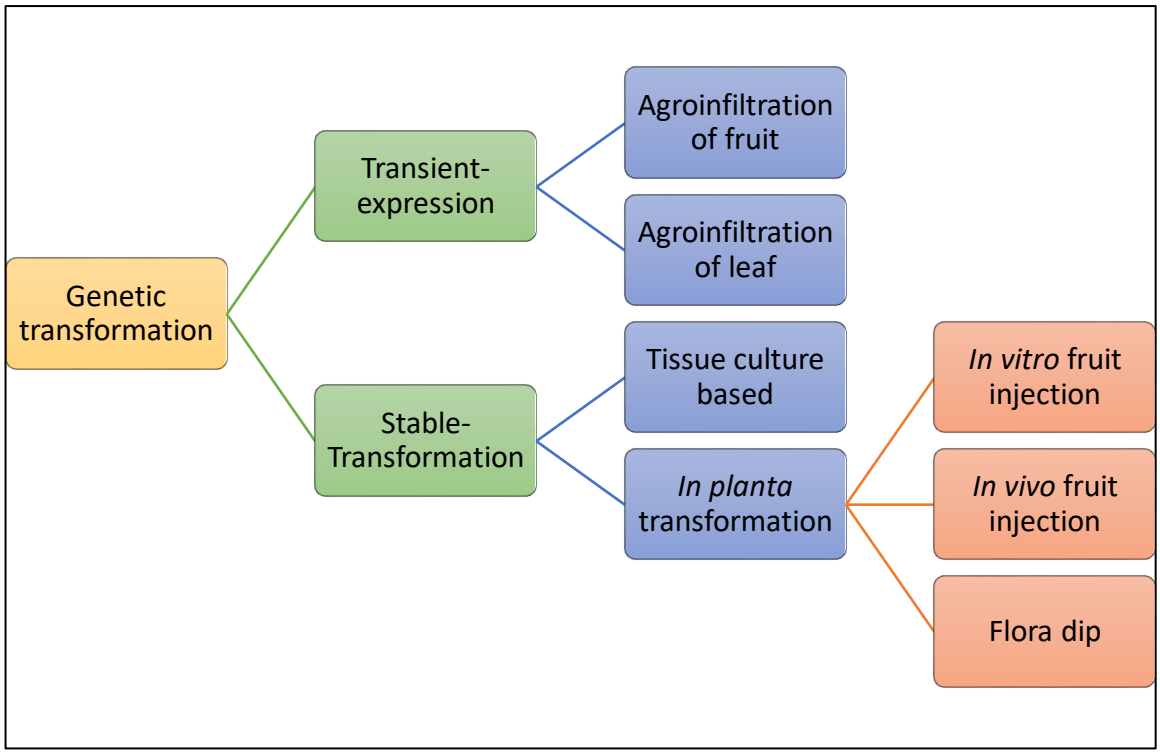

Figure 2.1: Different approaches used in transformation of tomato.

Genetic transformation can be defined as the insertion of foreign genes or DNA materials into the target plant cells in order to produce a transgenic plant with additional new genes. The production of transgenic plants for biological research is a routine in plant biotechnology. The genetic transformation protocols can be divided into two types (Figure 2.1) which are transient and stable transformations. Through genetic transformation, plant biotechnology has been successfully applied for developing abiotic stress resistance such as heat, drought and deficiencies and biotic stress resistance such as bacteria and fungi resistance (Islam et al., 2010). However, the gene expression analysis must be performed in order to confirm that the gene of interest is expressed stably and function well before its application to generating transgenic plant in the form of large scale (Solliman et al. 2016).

Genetic transformation is an important process in order to improve the target plant or to study the plant molecular genetics where the use of *Agrobacterium*-mediated transformation is common in order to transfer a foreign gene into the genome of a plant (Sun et al., 2015). Other than that, *Agrobacterium* act as an important tool in order to facilitate an improvement on genetic transformation along with other protocols such as agroinfiltration of leaves and fruits (Datta, 2015). There are two universally used strains of Agrobacteria involve in plant transformation which are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Ain 2013). In this review, only *Agrobacterium tumefaciens* was explained as it has been commonly used in genetic transformation protocols of tomato. A decade ago, *in planta* transformation study involved injection of *Agrobacterium tumefaciens* cells which carrying foreign T-DNA into fruit (Ahmad and Mirza, 2005). A reporter gene *B-glucuronidase* (*GUS*) is frequently used to demonstrate the location of shipment of T-DNA (Yasmeen al., 2008)

The main purpose of genetic transformation is to generate plant variety with desirable trait in terms of tolerant to abiotic and biotic stress so that the plant can survive and produce desirable amount of yield (Das et al., 2015). Usually, the production of the transgenic plant through genetic transformation cannot be achieved by conventional breeding. In addition, the genetic transformation may help in improve the unsatisfactory features in cultivars effectively and efficiently than conventional breeding (Mishra et al., 2017). The main reason for genetic transformation is for plant improvement in terms of abiotic and biotic stress where the transgenic plant more resistance to pest and disease, tolerate to herbicide, produce higher yield, able to survive in drought, hot, frosty and flood condition and

increase farm profit (Senapati, 2016). This can be concluded that genetic transformation approach in order to create tomato variety that tolerate to abiotic and biotic stress was better alternative to conventional breeding (Datta, 2015).

The most important prerequisite in genetic transformation in order to produce transgenic plant are the availability of a healthy target tissue, the protocols to transfer foreign gene into the cells and the method to select and regenerate transgenic plants at a satisfactory frequency level (Sah et al., 2014). The expand of knowledge from plant physiology must be combined together with molecular genetic transformation as this genetic transformation could become a powerful tool in order to produce transgenic plant rather than use conventional breeding (Cuartero et al., 2006). Although several genetic transformation protocols have been conducted to produce transgenic plant but these protocols have several problems such as low frequency of plant transformation which result in limiting the production of transgenic plants for commercial and large-scale usage (Hasan et al., 2008). *Agrobacterium* is a popular choice of researcher in genetic transformation protocols as it is an important tool to produce transgenic plant where it genetically transforms the host cells by transferring foreign gene into them (An et al., 2014).

2.3.1 History of *Agrobacterium*

Agrobacterium is a truly notable organism and has been recognized as the agent that causes the crown gall plant tumor. His research has revolutionized plant molecular genetics and brought about plant modification (Nester, 2015) A knowledge of the fundamental biology of the bacteria-plant interaction scheme made it possible to develop *Agrobacterium* as the main player in plant genetic modification. Braun (1958) recognized that some *Agrobacterium* product altered the characteristics of plant cells. These findings were very crucial because they offered useful hints as to the mechanism by which *Agrobacterium* transforms plant cells. Today, the product of *Agrobacterium* that affects the plant cell characteristics is known as DNA and this DNA is called T-DNA for transferring DNA from *Agrobacterium* to plant cells.

According to Nester (2015), in the 1960s, a number of laboratories skilled in bacterial genetics began to study the bacterial-plant system that led to the discovery of Ti plasmid where a piece of Ti plasmid, T-DNA, was transferred and randomly integrated into the plant cell chromosome. There are two sets map to the Ti plasmid where one set is the T-DNA (Garfinkel et al., 1981) and the other is *vir* genes that are required for the processing and transfer of T-DNA (Garfinkel and Nester, 1980). The *vir* genes on the Ti plasmid function in different phases of plant cell transformation. Furthermore, the *vir* genes are displayed only when the *Agrobacterium* is closely associated with a host plant and plant signals are acknowledged by the element of the regulatory scheme that is *vir* genes that activated other *vir* genes (Nester, 2015).

2.3.2 *Agrobacterium* and Plant Biotechnology

Agrobacterium-mediated transformation has created an amazing transgenic plant with significant characteristics ranging from resistance to disease, dietary content, and others. The first demonstration of that A. Barton et al. (1983) could use *A. tumefaciens* to produce transgenic crops which lead to the beginning of new knowledge in plant molecular biology research. The protocols have been established for *Agrobacterium*-mediated transformation and regeneration of many plants such as tomato (Yasmeen et al., 2008), soybean (Lee et al., 2013) and paddy (Sah et al., 2014). Transformation of crops not only by integrating foreign genes into plant cells, but also by regenerating transgenic plants from these genetically modified cells (Banta and Montenegro, 2008). It was thought impossible to generate transgenic monocot using *Agrobacterium*. But, now it is regarded as a routine for certain monocot cultivars (Banta and Montenegro, 2008).

Over the past two decades, a number of methods have been developed to improve the efficiency of *Agrobacterium*-mediated gene delivery such as microprojectile bombardment coated with agrobacteria. Another requirement for effective genetic modification is an effective selection method for plant cells with embedded T-DNA (Chung t al., 2006). Plant cells in which the foreign DNA was incorporated into the genome were chosen. (Walden and Wingender, 1995). Initially, the identification of transformed cells was accomplished by screening for nopaline production (Barton et al., 1983). But, several research papers given some changes to this original transformation scheme where the expression of antibiotic resistance genes such as neomycin phosphotransferase (*nptII*) from embedded

T-DNA in plant cells enabled the selection of transformed plant cells with antibiotic resistance (Banta and Montenegro, 2008).

2.3.3 *Agrobacterium*-mediated Transformation of Tomato

Different plant species have different gene transfer protocols depend which are suitable to in order to get high frequency level of transformation (Chaudhry and Rashid, 2009). *Agrobacterium*-mediated transformation is an efficient, effective and widely used system in order to transfer foreign DNA into dicotyledons plants such as tomato. Since 1907, the soil pathogen *A. tumefaciens* has been extensively studied as a source of a tumor inducing pathogen which permanently transformed plant cells to an active cell division. Currently, *A. tumefaciens* has become one of the most used method in plant transformation research in order to introduce foreign genes into target plant cells (Sah et al., 2014). The virulent strain of *A. tumefaciens* which induces crown gall disease consist a large mega plasmid which called as Ti plasmid and during the infection of *A. tumefaciens* on target plants, the segment of Ti plasmid which is T-DNA was transferred into the plant cell nucleus and get combined with the plant genome (Ozygit, 2012).

The *A. tumefaciens* used in genetic transformation process requires the presence of two important components which located on the Ti plasmid where the first important component is T-DNA and the second is the virulence region (Hwang et al., 2017). T-DNA which original part of the Ti plasmid is flanked by two borders that were classified as left border (LB) and right border (RB) (Kleinboelting et al., 2015). These two borders have the

sequence of 25 base pairs (bp) in length and the sequence are similar to each other (Combier et al., 2003). In addition, this T-DNA border is the only important part that needed to enable replacement of the original T-DNA sequences with desired DNA between left border and right border for genetic engineering in order to integrate this desired DNA with the genome of the plant (Tzfira et al., 2004). The right border is more active than the left as there is the presence of an enhancer close to the right border where the enhancer helps in unwinding the double stranded DNA at the right border (Ozygit 2012).

The T-DNA is located on a tumor inducing plasmid which consists others related important element of genes encoding and virulence genes that required for transfer mechanism of T-DNA into the target plant cells and some Ti plasmids contain one T-DNA while others contain multiple T-DNA region (Gelvin, 2003). During transferring process, this T-DNA region is released from the Ti plasmid by endonuclease and once this T-DNA has entered the target plant cells, it will go directly to the nucleus of the plant cells and bind into the host genome to ensure stable replication of the plant cells (John and Frandsen, 2011). The virulence (*vir*) genes which located on the Ti-plasmid affect the T-DNA transferring process to the plant cells where the transfer system was encoded by this *vir* genes and during T-DNA transfer, some *vir* proteins enter the plant cells in order to protect the T-DNA and facilitate its transport to the nucleus of the plant cells (Bundock and Hooykas, 1996). Large part from the activity of *vir* genes which carried by Ti plasmid was involved in the processing of T-DNA on Ti plasmid and also in the transferring process from this Ti plasmid into the target plant cells (Ozygit 2012).

Vir proteins encoded by *vir* genes play an important role in the *Agrobacterium*-mediated transformation process where each of these *vir* proteins have their own functions (Table 2.2) to process T-DNA and transfer it to the plant cells (Gelvin, 2003). There are eight *vir* genes located in the *vir* region of Ti plasmid which are *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F, *vir*G and *vir*H. Example, the induced of *vir*D1 and *vir*D2 proteins by *vir*D gene lead to the cutting of bottom strand at the T-DNA borders and release a single stranded T-DNA molecule from the Ti plasmid (Tzfira et al., 2004). In addition, *vir*A act as the sensor for phenolic substances and *vir*G act as phenolic response regulator and both of these proteins were induced by phenolic compound released by a wounded plant. Wounded plant is important for efficient transformation as the *Agrobacterium* can sense a wounded plant from these phenolic compounds that have potential for T-DNA transfer from the Ti plasmid to the plant cells through wounded place on the plant (Lee and Gelvin, 2008).

Table 2.2: *Vir* proteins and their functions.

(Source: Gelvin, 2003; Tzfira, 2004 Lee and Gelvin, 2008; Ozygit 2012)

Vir protein	Function
***Vir*A**	➢ Sensor for phenolic substances released by the wounded plant where this protein located in the membrane of *Agrobacterium*.
***Vir*G**	➢ Act as phenolic response regulator where it helps in increasing the level of transcription of the *vir* genes in order to produce *vir* proteins.
***Vir*B**	➢ Form the membrane channel or act as ATPases to provide energy required for T-DNA transfer process.
***Vir*C**	➢ Bind to the overdrive region and help to unwind double stranded DNA and activate DNA cleavage to generate T-DNA.
***Vir*D**	➢ Act as an endonuclease that releases T-DNA from Ti plasmid and plays a part in the integration of T-DNA with the plant genome.
***Vir*E**	➢ Shape the T-DNA into a transferable shape and form a pore on the plant membrane to promote the T-DNA pathway
***Vir*F**	➢ Interact directly or indirectly with T-DNA to form larger T-DNA complexes and regulate the cycle of division of plant cells.
***Vir*H**	➢ Improve transfer efficiency and neutralize certain plant compounds that influence bacterial growth.

2.3.4 T-DNA Transfer from *Agrobacterium* to Plant Cells

Virulence genes expression which produce virulence proteins leads to the production of a single stranded T strand which can be called as T-DNA that will be the transported into the plant cells and combine with the host genome (Sah et al., 2014). There are two proteins that function as two components sensory signal transduction regulatory system which are *Vir*A and *Vir*G proteins and both of these *vir* proteins located in the membrane of *Agrobacterium* (Ozygit, 2012). *Vir*A protein act as a sensory where it will detect the presence of plant phenolic compound that are induced on wounded plant while *Vir*G proteins act as phenolic response regulator where it will increase the level of *vir* genes expression in order to produce *vir* proteins that will process T-DNA and transfer it to the plant cells (Hwang et al., 2017). This can be concluded that *Agrobacterium* will recognize wounded plant cells through the release of phenolic compounds that act as inducer to activate *vir* genes expression which then attach to the wounded plant and export the T-DNA (Gelvin, 2003).

The right border will act as an initiation site for single stranded T-DNA synthesis which will then progressing leftwards and terminating at left border due to the *Vir*C protein binds to the overdrive region at the right end of the right border and helps in unwinding the double stranded T-DNA in order to produce single stranded T-DNA (Ozygit, 2012). Furthermore, *Vir*D1 and *Vir*D2 proteins that synthesized from *Vir*D genes will cleave both left and right T-DNA borders. In fact, *in vitro* experiments have been proved that the presence of *Vir*D gene is needed for the cleavage of double stranded DNA by *Vir*D2 protein (Hwang et al., 2017). Then, both *Vir*D2 and *Vir*E2 protein bind to the T-DNA in order to

protect the T-DNA from nucleolytic degradation that occur both in cytoplasm and in the nucleus that help in transferring the T-DNA across cell and supply specific targeting signals to the plant cell genome (Tzfira et al., 2004). This *Vir*E2 protein help in altering the T-DNA into a shape that can the nuclear pore of the plant cells.

After the single stranded T-DNA was produced, *Vir*B gene will induce the *Vir*B proteins in order to create a passage or the membrane channel to ensure the T-DNA can be transfer to the host plant cells and this *Vir*B protein also act as ATPhase to provide energy needed for T-DNA transfer process (John and Frandsen, 2011). Furthermore, *Agrobacterium* also harbors *Vir*H gene which this gene will produce *Vir*H protein that act as enzyme to detoxify of harmful phenolics compound released by the wounded plan (Ozygit, 2012). When the T-DNA already enters the plant nucleus, all the *vir* proteins that was attached to the T-DNA in order to facilitate the T-DNA transferring process need to be removed from T-DNA to allow efficient T-DNA integration with host plant cells genome (Lee and Gelvin, 2008). Finally, the *Vir*F protein interacted either directly or indirectly with the T-DNA to form larger T-DNA complexes in the plant nucleus and regulate plant cell division cycle (Hwang et al., 2017). The *Vir*B, *Vir*C, *Vir*D, *Vir*E and *Vir*F are involved in processing, transfer and integrate the T-DNA from *Agrobacterium* into the host plant cells.

2.3.5 The Application of *Agrobacterium* for Genetic Engineering Purpose

T- DNA is Defined as the T-region of the Ti plasmid that is loacted in the *Agrobacterium* and transmitted from the *Agrobacterium* to host plant cells to form crown gall tumors on the plant (Tzfira et al., 2004). The processing, transfer and integration of T-DNA from the *Agrobacterium* Ti plasmid into plant cells involving a big portion of the virulence activity of Ti plasmid-borne viruses (Gelvin, 2008; Ozygit, 2012; Hwang et al., 2017). Genetic experiments suggested that Ti plasmids were accountable for tumorigenesis where the T-DNA part of this plasmid was moved to host plant cells and incorporated with the plant cell genome. Although the Ti plasmid was used as an intermediate to introduce foreign gene into hos plant cells, the Ti plasmid's T-DNA area does not comprise a restrictive endonuclease site that no one can simply clone a gene of concern into the T-region (Hwang et al., 2017). In addition, the use of relatively small T-DNA binary vectors made it easier for researchers to analyze the transfer of non-T-DNA regions in hos plant cells (Ozygit 2012).

Although *Agrobacterium* capacity to transfer T-DNA region in host plant cells provides us with the use of *Agrobacterium* as a highly efficient model and a strong instrument for plant biotechnology to introduce foreign genes into target plants, but the following large-scale characteristics are tumor-inducing characteristics and the lack of endonuclease site limitation prevents its immediate use where some manipulation of Ti plasmid is needed before using *Agrobacterium* in genetic engineering (Hwang et al., 2017). A significant stage in the growth of T-DNA is the realization that the transfer of T-DNA to the host plant cells depended on the *vir* genes and the 25 base pairs of direct repeat structures marking

the T-DNA's left and right boundaries (Ozygit, 2012). A systematically altered Ti plasmid can be used as a mechanism for transferring T-DNA to the plant cell genome without causing cancers by deleting all wild-type T-DNA except for the left and right boundary sequences that enabled the creation of disarmed Ti plasmid when all wild-type T-DNA that induces tumors was removed (Lee et al., 2013).

There are significant barriers to be overcome in order to use *Agrobacterium* to produce transgenic plant which is the wild-type T-DNA on the Ti plasmid that has been removed to eliminate the pathogenicity of the bacterium resulting in the Ti plasmid being disarmed without influencing Ti plasmid's capacity to pass T-DNA into the nucleus of plant cells (Hwang et al., 2017). T-DNA must then include the interest gene and selection markers for transgenic crops. The Ti plasmid size is very large and usually tiny, making it quite hard to isolate and clone the Ti plasmid (Ozygit, 2012). In order to solve these problems, most scientists use a binary vector scheme where the T-DNA region is performed on a broad-host replication and the viral genes needed for T-DNA transfer are situated on disarmed Ti plasmid where this binary vector scheme has offered scientist flexibility and increased manufacturing of transgenic plants (Hwang et al., 2017).

Traditional transformation such as electroporation protoplast transformation, microinjection or polyethylene glycol fusions (Table 2.3) is not appropriate for transgenic plant production because protoplast plant regeneration is time-consuming and low effectiveness. Microprojectile bombardment is the most important alternative to Ti plasmid T-DNA delivery system for plants where its spherical tungsten particles are coated

with DNA and accelerated to high speed with a particle piston such as DNA penetrate the plant tissue (Ozygit, 2012). This shows that the microprojectile bombardment can be used to introduce foreign gene into a broad spectrum of plant species. However, the bombardment method tends to cause DNA insertion multiplying duplicate and the loss of DNA molecular integrity (Hwang et al., 2017). The *Agrobacterium*-mediated transformation is therefore still the most frequently used and the most common method for transgenic plant generation.

Table 2.3: Comparison between different types of plant transformation methods.

(Source: Hwang et al., 2017; Sah et al., 2014; Ozygit, 2012)

Method	Pros	Cons
***Agrobacterium*-mediated transformation**	• Easy to set up and use. • Less expensive • Higher stability of transferred gene.	• Only suitable for dicot plants. • Several monocot and woody plants are not compatible.
Microprojectile bombardment	• Efficient system for transient expression. • A wide range of plants can be used.	• Multiple copies of DNA insertions. • Loss of molecular integrity of DNA insertions.
Electroporation (Protoplast)	• Convenient. • Simple.	• Limited to few plant cell types. • Difficult to regenerate viable plants.
Microinjection (Protoplast)	• Fast. • Only few successful reports. Example is barley.	• Limited to few plant cell types. • Difficult to regenerate viable plants.
Polyethene glycol (Protoplast)	• Easy to perform with relatively low cost.	• Limited to few plant cell types. • Difficult to regenerate viable plants.

2.4 Transient Expression

Agrobacterium-mediated transformation is more preferred method in transient transformation as it provides safe and very rapid gene expression and the expression part containing gene of interest which is carried by *Agrobacterium* in its T-DNA region on Ti plasmid (Hasan et al., 2008). In addition, transient expression is a simple and effective method of transferring gene of interest into plant cells for the rapid production of recombinant protein. According to Orzaez (2006), to shorten the time for gene functional analysis in fruit, transient expression method was developed in order to express foreign gene rapidly. Other than that, transient expression is an easy, rapid and efficient protocol for genetic transformation of tomato as it is not a time consuming as compared to tissue culture that need tissue culturing phase and also not a lengthy gene manipulation process like stable transformation method (Datta, 2015)

Furthermore, *Agrobacterium*-mediated transient transformation is a quick alternative to assay gene function, promoter behavior and protein function when generation of a transgenic plant from stable transformation is unnecessary. But, plant defense reactions also play a main role in reducing transient transformation. (Hwang et al., 2017). In the host plant nucleus, the single stranded T-DNA can be processed into double stranded T-DNA which is readily translated without genome integration (Wrobleski et al., 2005). Since integration is not the primary objective of the transient scheme, a plant selectable marker is generally not needed, the drawback of which is the extremely variable transformation for each *Agrobacterium* strain, target plant species and specific tissues (Hwang et al., 2017). The method of transient expression is known as agroinfiltration where this

infiltration method required the process in injection of *Agrobacterium* cultures, for example, injection through the stylar apex of fruit (Hasan et al., 2008).

2.4.1 Agroinfiltration of Fruit

Orzaez et al., (2006) reported *Agrobacterium*-mediated transformation method to study the promoter activity assisted by reporter genes using tomato fruits via agroinfiltration of fruit protocol. Progression of the infiltration on the tomato fruits were monitored with *Agrobacterium* cultures stained with methylene blue. The tomato fruits were injected with a 1 m-L syringe through the stylar apex with 600 µL of infiltration medium containing methylene blue stained bacteria and the infiltration solution reach the entire surface of fruit surface. Once the infiltration was confirmed, the tomato fruit was injected with a double-reporter plasmid containing Yellow Fluorescent Protein (YFP) and *β-glucuronidase* (*GUS*) directed by 35S promoter. After 4 days, high levels of YFP were clearly visible around the placenta of the fruits under UV light. However, strong YFP and moderate *GUS* also found in the pericarp due to higher sensibility of the method as the fruits tissue susceptible to *Agrobacterium* infection. This observation indicate that reporter genes can be efficiently expressed in fruit via agroinfiltration of fruit.

Hasan et al., (2008) investigated the agroinfiltration of fruit mechanism on ripen tomato fruits by performing transient *GUS* assay in order to observe transient expression of *GUS* under the control of 35S promoter on various incubated period and fruit tissues. Transverse sections of fruit were observed to analyze transient *GUS* expression in different tissues of the fruits. A range of percentage of transient *GUS* expression was observed for different

incubation periods and tissue type combination. The highest value of 86.67% transient *GUS* expression was observed for the tip of the ripened fruit incubated for 48 hours while the lowest value of 53.53% was observed for the base of the fruit incubated for 96 hours. Maximum transient *GUS* activity was observed in tomato fruits incubated for 48 hours as the transient assay are usually performed 48 hours after agroinfiltration. Agroinfiltration of ripen fruits of tomato showed an excellent protocol for transformation not only positive result but also showed very high efficiency of transformation.

Hoshikawa et al., (2018) improved the efficient transient expression in tomato fruits using agroinfiltration-mediated high expression system. Innovation and improvement of effective tools for transient expression in plant cells is crucial for the development of plant biotechnology. The agroinfiltration was performed with the binary vector pBYR2HS-EGFP. The suspension was slowly infiltrated into the fruits tissue from the tomato stylar canal by using a 1-mL syringe needle. This complete agroinfiltrated fruits were maintained at room temperature for 3 days before the Green Fluorescent Protein (GFP) was observed under blue LED lamp. GFP fluorescence was detected in tomato fruits infiltrated with *Agrobacterium* containing the binary vector pBYR2HS-EGFP. The expression level of GFP was especially high in the placenta of fruits. This showed that transient expression system using pBYR2HS is an effective system and it enhanced the transient expression level in tomato fruits.

2.4.2 Agroinfiltration of Leaf

Wroblewski et al., (2005) conducted a research on optimization of *Agrobacterium*-mediated transformation of transient expression on tomato leaves. 24 *Agrobacterium* strains were evaluated to increase the diversity of strains available for transient expression. The *Agrobacterium* was diluted to densities of OD_{600} = 0.4 – 0.5 to avoid weak transgene expression and leaves wilting. The pressure was applied on the bacterial suspension against the lower side of a young leaf lamina with a syringe-free needle. In tomato leaf, the bacterial penetrated the tissue easily and the infiltrated regions were usually circular or irregular shape due to the restriction by the major leaf veins. The intensity of *GUS* staining reached its maximum within 4 – 5 days after agroinfiltration. Many of the tested *Agrobacterium* strains induced high level of *GUS* expression despite severe necrosis in the infiltrated areas. As a result, high levels of transient expression in tomato leaves were achieved. The level of transient expression observed often exceed the levels of expression in stable transformation.

Chen et al., (2013) reported agroinfiltration as an effective strategy of gene delivery into plant cells. *Agrobacterium* strains with optimal concentration OD_{600} = 0.12 was used in order to meet the needs for maximum transgene delivery without causing tissue necrosis and cell death. A syringe-free needle was used to introduced *Agrobacterium* into plant leaves. A small nick was created with a needle in the epidermis on the back side of the leaf and the infiltration medium was injected into the leaf through the nick by using syringe-free needle. As the *Agrobacterium* enter the intercellular space of the leaf, the light green color begins to darken indicating a successful infiltration. High level of transgene expression through agroinfiltration have been achieved in comparison to the stable

transgenic plant. The simplicity of agroinfiltration allow a quick assessment on the expression level of interest gene in plant cells under established condition. The short timeline and higher expression level make transient expression an interactive protocol for genetic transformation of plants.

Zhao et al., (2017) improved the syringe agroinfiltration protocol to enhance transformation efficiency by combinative chemical of 5 – Azacytidine, Ascorbate Acid and Tween-20 within plant cells. The *GUS* expression and *GUS* activity were measured to determine the transformation efficiency. The result showed that 20 µM Azacytidine, 0.56 mM Ascorbate Acid and 0.03% Tween-20 is the optimal concentration that could significantly improve the transformation efficiency of agroinfiltration and this concentration combination could increase the transformation efficiency to a greater extent. This showed that all those chemicals played roles in enhancing the expression of gene which affects the transformation efficiency of agroinfiltration and at the same time can improve the expression of gene at a certain level. Thus, an optimized agroinfiltration was developed which might be a great method in transient transformation. The improvement of agroinfiltration was performed which will be useful in transient transformation study of genetic engineering.

2.5 Stable Transformation

Significant progress has been made in the genetic transformation of tomatoes in latest years, and this genetic transformation is a basic step in the production of transgenic plant and plant improvement. As normal, *Agrobacterium*-mediated transformation was used to transfer the gene of interest in tomato plants. According to Sun et al., (2015), before this, they have developed an *Agrobacterium* transient genetic expression transformation but unfortunately no gene was integrated into the genome of tomato plant as this transient gene were unstable and become lost through environmental factors. Thus, expressing of gene of interest into tomato plant is a candidate approach. Sun et al., (2015) mentioned that, in their experiment, the transgenic tomato that they produced was considered successful on the basis of its capability to carry gene of interest. However, the efficiency of stable transformation is affected by many factors such as *Agrobacterium* strains, inoculation duration and other antibiotics in the media and plant genotype (Safdar and Mirza, 2014)

Furthermore, stable genetic transformation is often a lengthy method requiring a protocol to regenerate whole plants from transformed cells or tissues. The T-DNA must be integrated into the plant cell genome to ensure that the gene can subsequently be transferred to the next generation (Sah et la., 2014). The use of reporter genes enables transformed plant cells to be quantified where reporter gene products such as *β-glucuronidase* (*GUS*) and Green Fluorescent Protein (GFP) are frequently used. The GFP is a useful *in vivo* marker for examining gene expression because it can be excited with either ultraviolet or blue light (Hwang et al., 2017). Various antibiotic resistance genes have been placed into the T-DNA region of binary vectors used for transgenic plant

selection where kanamycin and hygromycin resistance genes are the most commonly used selectable marker genes in transgenic plant selection where it is essential to identify foreign genes that have been incorporated into plant chromosomes (Lee at al., 2013).

2.5.1 Tissue Culture-Based

Shahriari et al., (2006) reported tissue culture based of *Agrobacterium*-mediated transformation in order to study effect of tomato regeneration on different types of media. *Agrobacterium* strain harboring the binary vector pBII21 was used. Cotyledon and hypocotyls were used as explants. Growth regulators BAP, NAA and Zeatin were used. Explants were subcultured every 2 weeks and after 4 weeks of culture, the percentage of shoot formation was determined. Then, after 5-6 weeks, elongated shoots were excised and placed on shoot elongation medium where most of these elongated shoots formed roots MS basal medium lacking growth regulators. The maximum shoot regeneration for cotyledon was observed in MS media containing 2 mg L^{-1} Zeatin. While for hypocotyl, the high frequency of regeneration was observed in MS media containing 2 mg L^{-1} BAP or 2 mg L^{-1} Zeatin combined with 0.1 mg L^{-1} NAA. The type of plant hormone used is a crucial factor controlling tomato shoot regeneration. The result showed the effect of zeatin on regeneration efficiency was more significant among other plant hormone regulators as zeatin stimulates the organogenesis of tomato explants such as leaves.

Devi et al., (2012) considered the tissue culture-based protocol as a simple and reproducible protocol for obtaining high frequency of transformation in tomato. Factors governing the efficiency of *Agrobacterium*-mediated transformation include age of

explants, bacterial concentration, pre-culture period and co-cultivation duration were optimized. The explants were cultured on MS medium supplemented with BAP (2.0 mg L^{-1}) and kinetin (1.0 mg L^{-1}). Disarmed *Agrobacterium* strains which carry the binary vector contained *GUS* under the control of 35S promoter was used. The result showed that the 10 days old seedling explants were proven to be the superior explant for transformation over 7 and 12 days old. The effect of 1 day pre-culture was also significant over 2 and 3 days. Furthermore, The *Agrobacterium* with density OD_{600} = 1 gave maximum *GUS* expression on treatment duration of 30 minutes. The *Agrobacterium*-mediated transformation through tissue culture-based protocol can produce high frequency of transformation in tomato.

Ma et al., (2015) investigated five factors influencing transformation efficiency which are stage of explants, preculture, *Agrobacterium* density, infection time and co-cultivation period with an objective to develop a high throughput transformation protocol for tomato. *Agrobacterium* strains were suspended in a liquid medium on a plate and density was set to different optical densities. The cotyledon and stem explants were excised and infected with *Agrobacterium* strain by gently swing the plate. Then, both explants were transferred to selection medium for cotyledons and stem. The explants were subcultured every four weeks. After eight weeks, the shoots were excised and transferred to rooting medium. The result showed that the optimal protocol for cotyledon explants consisted of 8-9 days age of seedling, 2 days of preculture, an *Agrobacterium* of OD_{600} = 0.6, 3 days of co-cultivation and an infection medium of 20 minutes. For stem explants, consisted of 4-5 days age of seedling, 3 days of preculture, an *Agrobacterium* suspension of OD_{600} = 0.6, 4 days co-

cultivation and infection time of 15 min. The age of seedling, the use of different plant tissue and different tomato genotype play an important role in tomato transformation.

2.5.2 *In Vitro* Fruit Injection

Yasmeen et al., (2008) investigated *in planta* transformation method through *in vitro* Fruit injection. The *Agrobacterium* carrying binary vector pROKIIAP1GUSint for Apetala 1 (*AP1*) gene, pROKIILFYGUSint for Leafy (*LFY*) gene and p35SGUSint for *B-glucuronidase* (*GUS*) reporter gene was used. The *Agrobacterium* was injected into tomato fruit by using 1 mL sterile syringe and incubated on different period. The result showed that the fruit injected with pROKIIAP1GUSint showed 17% transformation, pROKIILFYGUSint showed 19% transformation and p35SGUSint showed 21% transformation. The highest frequency of tomato transformation was obtained following 48 hours incubation. This result showed that 48 hours is the optimum incubation period followed by seeds of the fruit incubated for 36 and 72 hours. That seedling survival decreases with an increasing incubation period. *Agrobacterium* required a limited time to achieve high level of transformation rates.

Safdar and Mirza (2014) conducted an experiment on *Agrobacterium*-mediated transformation through *in vitro* injection protocol on tomato fruits. The objective of this experiment was to assess *in vitro* transformation procedures in tomato fruits by using *Agrobacterium* strain carrying *HAL 1* gene and *NPTII* gene. The *in vitro* protocol involved the injection of *Agrobacterium* suspension into tomato fruits for different incubation periods. The *Agrobacterium* strains LBA4404 with optical density $O.D_{600} = 0.8$ was used.

Seeds were collected from *Agrobacterium* injected fruits and inoculated on selection medium which is MS medium containing 100mg/l kanamycin. The result showed that the germination percentage was high for 48 hours incubation period followed by 24 hours while 6 and 10 hours incubation period had no prominent impact on stable transformation efficiency. This indicates that most of the T-DNA insertion events in fruit seeds occur at two days incubation period.

2.5.3 *In Vivo* Fruit Injection

Yasmeen et al., (2008) investigated *in planta* transformation method through *in vitro* Fruit injection. The *Agrobacterium* carrying binary vector pROKIIAP1GUSint for Apetala 1 (*AP1*) gene, pROKIILFYGUSint for Leafy (*LFY*) gene and p35SGUSint for *B-glucuronidase* (*GUS*) reporter gene was used for plant transformation. The *Agrobacterium* was injected into tomato fruit by using 1 mL sterile syringe. The influence of mature (red color) vs immature (green color) fruits was evaluated. The result showed that mature red fruit gave a higher transformation percentage where 40% on pROKIIAP1GUSint. 35% on pROKIILFYGUSint and 42% on p35SGUSint. While immature green fruit gave a lower transformation percentage where 2% on pROKIIAP1GUSint. 5% on pROKIILFYGUSint and 5% on p35SGUSint. The high percentage of transformation in mature red fruit due to the easier penetration of fruit by the *Agrobacterium* as the fruit loss of cell to cell contact during fruit ripening which help in the penetration of the *Agrobacterium*.

Safdar and Mirza (2014) conducted an experiment on *Agrobacterium*-mediated transformation through *in vivo* injection protocol on tomato fruits. The objective of this

experiment was to assess *in vivo* transformation procedures in tomato fruits by using *Agrobacterium* strain carrying *HAL 1* gene and *NPTII* gene. This *in vivo* protocol involved simple injecting bacterial culture into intact tomato fruits. The *Agrobacterium* strains LBA4404 with optical density $O.D_{600}$ = 0.8 was used. Transformed plants were selected on 100 µg/mL *kanamycin* and final selection of transformed plant was assessed on the basis of Polymerase Chain Reaction (PCR). The result showed that out of 1100 seed, only 18 were found to be resistant to antibiotic with transformation rate 0.0018%. Low rate of *in vivo* transformation might be due to low temperature as the condition of treatment not more than 15^{o}C where this temperature is not suitable for bacterial survival on treated tomato plants. This low temperature also might prevent the T-DNA insertions in the injected fruits.

2.5.4 The Floral Dip

Yasmeen et la., 2008 investigated *in planta* transformation of tomato through the floral dip method. Both unopened flowers before pollination and open flowers after pollination were used for transformation. The *Agrobacterium* carrying binary vector pROKIIAP1GUSint for *Apetala 1* (*AP1*) gene, pROKIILFYGUSint for *Leafy* (*LFY*) gene and p35SGUSint for *B-glucuronidase* (*GUS*) reporter gene was used for plant transformation. After treatment, the flowers were kept under observation until fruiting. The result showed that flowers treated before pollination gave higher percentage of transformation compared to those treated after pollination. Furthermore, the flowers treated with pROKIIAP1GUSint was not survived and failed to set fruit. However, flowers transformed with pROKIILFYGUSint was survived and able to set fruit. In addition, it was observed that

the overexpression of flowering genes had negative effects which resulted in the failure of fruit formation in the case of *AP1* gene.

Safdar and Mirza (2014) conducted an experiment on *Agrobacterium*-mediated transformation through *in vivo* injection protocol on tomato fruits. The objective of this experiment was to assess floral dip transformation procedures in tomato fruits by using *Agrobacterium* strain carrying *HAL 1* gene and *NPTII* gene. This floral dip protocol involved simple injecting bacterial culture into intact tomato flowers. The *Agrobacterium* strains LBA4404 with optical density $O.D_{600} = 0.8$ was used and it was resuspended in 4 mL of pollen germination medium to make a thick solution. The result showed that from 25 injected flowers, only 17 flowers were found healthy reaching maturity stage and the rest of the flowers withered. In addition, the fruits produced from these flowers also showed abnormal and stunted development. Out of 1100 seed assayed, only 18 were found to be resistant to antibiotic with transformation rate 0.0018%. The physiological state of the plants and the concentration of *Agrobacterium* can all affect the *in planta* transformation frequency.

2.6 Comparison between Tissue Culture-Based and *In Planta* Methods

Datta (2015) conducted an experiment to compared different transformation method between tissue culture-based and *in planta* transformation. Table 2.4 shows different parameters of tissue culture-based and *in planta* transformation for stable transformation were compared to find out the most suitable of stable transformation method for tomato. Stable *Agrobacterium*-mediated transformation is both tissue-culture based and non- tissue culture based which is *in planta* transformation where in the present study both transformation approaches were utilized. A comparison of the transformation processes in tomatoes showed differences in transformation efficiency and transformant regeneration. From the table, the tissue-based transformation of cotyledon leaves was established to be time consuming and needed a big number of explants resulting in few transformed lines which result in low transformation frequency. In contrast, a non-tissue culture-based was established to provide putative transformed seedlings with a greater proportion of transformants in a short time, requiring a lower number of explants to acquire transformed seedlings. Therefore, *in planta* transformation is more effective compared to tissue culture-based transformation.

Table 2.4: Comparison between tissue culture-based and *in planta* transformation.

(Source: Datta 2015)

Parameters	**Tissue culture-based**	***In planta***
Explant	Cotyledon leaves	Whole seed
Optical Density OD_{600}	0.6 – 0.8	1.2 – 1.4
Incubation time	30 min	30 min
Co-cultivation period	24 hours	24 hours
Number of regenerated shoots	2.30±0.07	3.33±0.2
Time required for shoot development	65 days	35 days
High regeneration efficiency in selection (%)	25 – 35	92 - 95
Time to get transformed plant	No plantlet regenerated	65 – 70 days
Bioassay	Antibiotic selection 50 mg/l kanamycin	*GUS* assay, antibiotic selection

2.6.1 Punctured-Hypocotyl for Efficient Tissue Culture-Based Method

Sivankalyani et al., (2014) investigated the tissue culture-based method in order to identify the highly efficient transformation of tomato. The effectiveness of the punctured-hypocotyl and ordinary immersion method of *Agrobacterium*-mediated transformation by using hypocotyl explants were compared. In the punctured-hypocotyl transformation, sterile hypodermic needles were touched deep in the suspension of *Agrobacterium* fortified with 100 μM acetosyringone and the hypocotyl explants were punctured roughly 1.5 mm in depth and 6 to 8 times randomly throughout the explants using the corresponding needles. While in the immersion transformation, hypocotyl explants without puncture were completely immersed in the *Agrobacterium* suspension. Transformation factors such as bacterial density OD_{600} = 0.4, 48 hours of co-cultivation and 40 minutes of infection were ideal for maximum transformation efficiency in tomato. The outcome shows that the transformation efficiency in tomatoes was higher with the punctured-hypocotyl method than the immersion method and relatively higher than the earlier reported transformation efficiency such as *in vitro*.

Hypocotyl was discovered to be the most responsive explant for increasing transformation frequency than tomato cotyledon and leaf explants (Gubis et al., 2004). This has shown that the sort of explant plays a significant part in transformed plant manufacturing (Bhatia et la., 2004). Previously, it has been reported that the wounding site of host plant cells released phenolics substances that aid in the induction of *vir* genes in the *Agrobacterium* resulting in the transferring of T-DNA from the *Agrobacterium* into the host plant cells (Lee and Gelvin, 2008). Therefore, puncturing hypocotyl explants and infecting them with

Agrobacterium most probably exposes the competent cells to *Agrobacterium* infection and improves transformation efficiency (Sivankalyani et al., 2014). In general, explant wounding during transformation offers a way for the *Agrobacterium* to move deep into the plant tissue to infect the meristematic cells which result in higher transformation efficiency. The method that had been developed is easy and effective and can be used to pass desirable genes into tomatoes for future enhancement in terms of quality and amount and as a suitable alternative for the development of abiotic and biotic stress tolerant plants (Bhatnagar-Mathur et al., 2008).

2.7 Factors Affecting the Transformation Efficiency of Tomato

According to Ma et al., (2015), there are five factors that influencing transformation efficiency which are cultivar, *Agrobacterium* density, co-cultivation duration, infection time and pre-culture. The first factor is cultivar where different cultivars showed different transformation frequency where the transformation frequencies were acquired from 6% in cv Pusa Ruby (Vidya et al., 2000), 13% in cv Rio Grande (Safdar and Mirza 2014) and 40% in cv Micro-Tom (Sun et al., 2006). The second factor is *Agrobacterium* suspension as its concentration had the strongest effect on transformation where different *Agrobacterium* optical densities have been recorded in multiple tomato cultivars (Qiu et al., 2007; Sun et al., 2006). The third factor is co-cultivation time where 3 days for cotyledon explants and 4 days for stem explants was suitable time for co-cultivation (Ma et al., 2015). The fourth factor is infection time where the optimum time to dip the bacterial suspension was 20 minutes for cotyledon explant and 15 minutes for stem explants (Ma et al., 2015). The fifth factor is pre-culture where 2 days for cotyledon explants and 3 days for stem explants acquired the greatest transformation frequency (Ma et al., 2015).

CHAPTER 3

CONCLUSION AND RECOMMENDATIONS

From the experiment conducted by Yasmeen et al., (2008), it can be concluded that the injection of ripened tomato fruit through *in planta* transformation gave an outstanding transformation efficiency. In the study of *in vitro* fruit injection, a 48-hour incubation period was discovered to be optimal. While experiments with *in vivo* fruit injection, applications of *A. tumefaciens* showed excellent results to mature fruits than immature fruits. Furthermore, flowers before pollination gave a greater rate of transformation. *In vivo* yielded the greatest transformation result among three protocols that are *in vitro, in vivo* and floral dip transformation. However, through floral dip protocol, the overexpression of the following genes had adverse effects on *in planta* transformation resulting in the failure of fruit formation. In addition, despite the flowering time of transgenic crops *LFY* and *API* and the transgenic plants formed brief stems, the flowers did not shed ordinary pollens and failed to produce fruit.

Comparing the result of tissue culture-based transformation with protocols of *in planta* transformation from the experiment undertaken by Safdar and Mirza (2014), it can be concluded that the method of transformation of *in vitro* fruit injection is much easier, more efficient and more economical to obtain transgenic plants. In addition, *in vitro* fruit transformation merely avoids the use of costly hormones such as zeatin, various media formulations and big infrastructure as used in the tissue-culture studies. The 48-hour

incubation period showed the highest amount of transformants as further increases in the incubation period slow down the introduction of T-DNA resulting in reduced transformation frequencies. In conclusion, the developed *in vitro* fruit injection is a straightforward and effective method of transformation of planta that showed 42% of the frequency of transformation. However, the physiological state of the crops, the medium of infiltration and the concentration of *Agrobacterium* are also variables that can all influence the frequency of transformation of *in planta*. It may be feasible to improve effectiveness and simplify the protocol by optimizing those variables.

In the experiment of tissue-culture based conducted by Sivankalyani et al., (2014) which comparing punctured-hypocotyl explant with immersion method (without puncture), it can be concluded that transformation efficiency was higher with the puncture-hypocotyl method resulting in transformation efficiency of 55.8 percent compared to the immersion method resulting in transformation efficiency of 48.3 percent. In this experiment, the combination of treatments which are *Agrobacterium* density OD_{600} = 0.4, 40 minutes infection time, 48 hours co-cultivated period and *Agrobacterium* strain LBA 4404 was discovered to be an ideal condition for increasing transformation efficiency. The optimum length of *Agrobacterium* infection and co-cultivation enhances the attachment of *Agrobacterium*, which helps to effectively transfer foreign genes into the target tissue. The punctured-hypocotyl explant may be a straightforward method that can enhance the transformation efficiency of tomato. In conclusion, the protocol of punctured-hypocotyl is the most efficient tissue-culture based transformation method that resulted in 55.8% of transformation efficiency.

In a very brief period of time, plant biotechnology has evolved into a distinct scientific discipline and *Agrobacterium*-mediated transformation has lately emerged as an effective method for plant genetic manipulation. Although other method of DNA transfer are also being used such as microprojectile bombardment, *Agrobacterium*-mediated transformation has significant benefits over these technologies in terms of increasing transformation frequency and others (Ozygit, 2012). Transgenic plants of many economically significant plant species or elite varieties such as *BT* maize have been created using *Agrobacterium*-mediated transformation method. This scenario is relevant because crops are the main and most significant source of our nutrition. James (2010) stated that the worldwide region of transgenic crops continues to grow for the fifteenth successive year. Although there are many debates about transgenic crops and transgenic method, the quantity and range of transgenic crops will improve in the future in line with the growing wild population and demands (Ozygit, 2012).

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