Stable Transformation of *Solanum lycopersicum* L. With OmpK Antigen Using Hygromycin as Selective Agent

By

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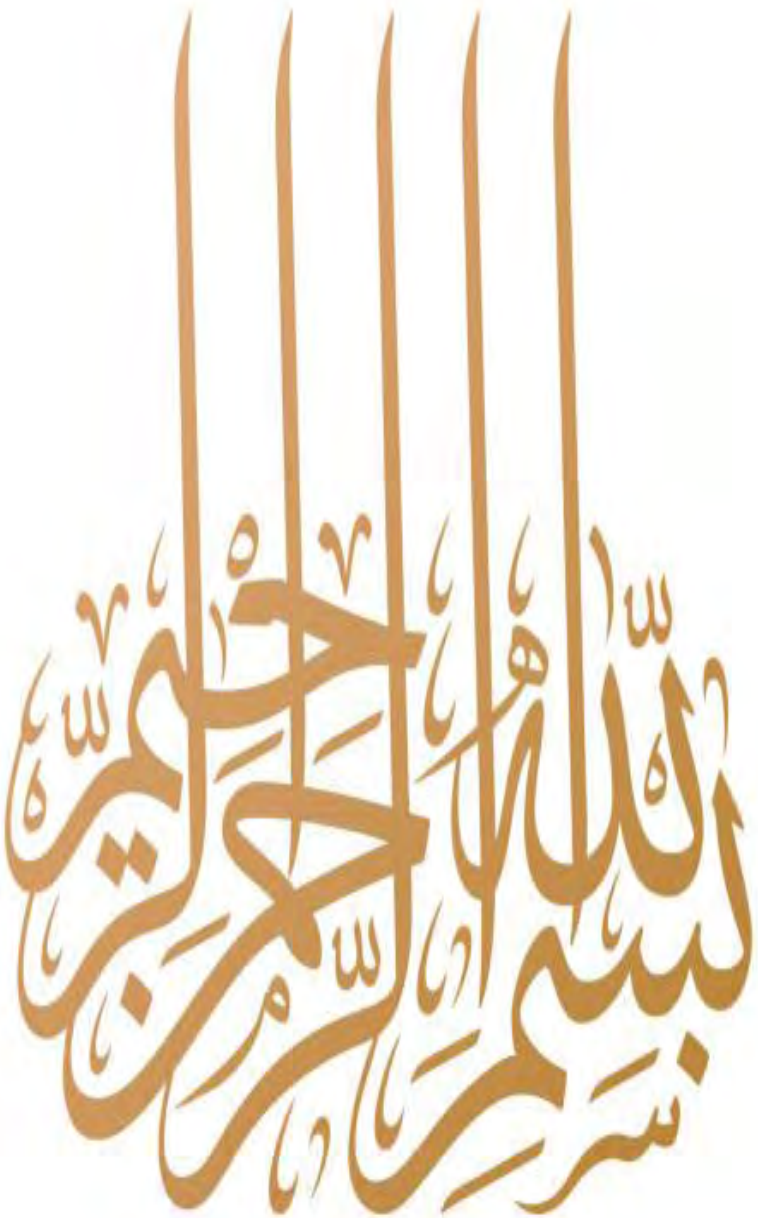
بسم الله الرحمن الرحيم

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CERTIFICATE

This thesis, submitted by **Ms. Fizza Ahmad Tariq** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Dated: **October 20, 2023**

Declaration of Originality

I hereby declare that the work presented in this thesis is the result of my own effort and research work, carried out in the Plant Biotechnology Lab, Department of Biochemistry, Quaid-I-Azam University, Islamabad.

This thesis is my composition; no part of it has been presented for any degree previously, nor does it contain, without proper acknowledgment, or reference, any material from the published resources to the best of my knowledge.

Fizza Ahmad Tariq

This thesis is Whole-heartedly
dedicated to
My Loving Parents
For their endless love, affection,
support, and courage

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
BSA	Bovine Serum Albumin
°C	Degree centigrade
µl	Micro liter
µm	Micrometer
2,4-D	2,4-dichloro phenoxy acetic acid
BAP	6-Benzylamino purine
BC	Before Christ
bp	Base pair
cm	Centimeter
CTAB	Cetyl trimethyl ammonium bromide
Da	Daltons
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo-nucleic acid
dNTP	Deoxynucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
F	Forward
FAO	Food and agriculture organization
g	Gram
ϒ	Gamma
HCl	Hydrochloric acid
HEPA	High-Efficiency Particulate Absorption
HRP	Horse-radish peroxidase

IAA	Indole acetic acid
IBA	Indole butyric acid
ICBN	International Code of Botanical Nomenclature
km	Kilometer
LB	Luria Broth
LA	Luria Agar
LDL	Low-density lipoprotein
LFH	Laminar Flow Hood
Mb	Mega bases
mg	Milligram
mg/ml	Milligram/milliliter
mg/mol	Milligram/mole
Mg^{+2}	Magnesium ion
mM	Milimole
MS	Murashige and Skoog
N	Normal
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
NLS	Nuclear localization signal
O.D	Optical density
OmpK	Outer Membrane Protein K
OMPs	Outer membrane proteins
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol method

PGRs	Plant growth regulators
pH	Power of hydrogen ions
Psi	Pounds per square inch
qRT-PCR	Quantitative real-time PCR
R	Reverse
RB	Right border
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPS	Relative percent survival
SH	Schenk and Hildebrandt medium
SQend	Starting quantity of endogenous gene
SQtrans	Starting quantity of transgene
T4SS	Type IV secretion system
T-DNA	Transfer deoxy ribonucleic acid
Ti	Tumor-inducing
TMB	3,3',5,5'-Tetramethylbenzidine
TSE	Tomato seed extract
TSP	Total soluble protein
UV	Ultra-violet

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ABSTRACT

Vibriosis, caused by *Vibrio anguillarum*, is a lethal bacterial infection that results in hemorrhagic septicemia and the death of various fish species in aquaculture as well as fish farms. It accounts for about 50% of the fish species' death and has been associated with significant economic losses in the aquaculture sector globally. Hence, there is a dire need to control vibriosis. Due to the drawbacks of using antibiotics and other drugs to treat vibriosis, vaccination is an effective method. OmpK from *Vibrio anguillarum* can be used as a potential vaccine candidate. Plants can be used as the expression system for the production of subunit vaccines as they have many advantages over other expression systems. The present research work aimed to optimize hygromycin concentration for the selection and to develop an efficient stable *Agrobacterium*-mediated transformation protocol for *Solanum lycopersicum* L. cv. Rio Grande, and to express OmpK antigen. The conditions for seed sterilization, germination, and tomato explants regeneration were tested. Promising results were obtained when seeds were germinated on half MS media after being sterilized with 0.1% mercuric chloride. Zeatin at a concentration of 2mg/L produced good regeneration efficiency. *Solanum lycopersicum* L. explants were treated with different concentrations of antibiotic hygromycin, and 25mg/L was found as the optimal concentration for the selection of transformed nodal explants. Successful transformation with OmpK antigen was carried out, in which 10 minutes of the infection time and 2 days of co-cultivation time showed the highest transformation efficiency of 60%. PCR was used to confirm the successful transformation using gene-specific primers. Transgene expression in transgenic tomato explants was analyzed by quantitative real-time PCR (qRT-PCR) in comparison to the β-actin gene. Further, protein expression was confirmed through Dot blot, Western blot, and ELISA. Taken together, the successful stable transformation of *Solanum lycopersicum* L. with *OmpK* may facilitate the development of a cost-effective subunit vaccine against vibriosis in fish species.

Keywords: *Solanum lycopersicum* L., OmpK, Vibriosis, Aquaculture, *Agrobacterium*-mediated transformation, Hygromycin, qRT-PCR, Western blotting, ELISA

INTRODUCTION

1.1. Global importance of marine life

About 70% of the Earth's surface is made up of water, out of which more than 97% is covered by the ocean (Skuse *et al*., 2021). The oceans have great importance in human life. On one side where they have a cultural value that gives peace and joy, on the other side they provide a vast ecosystem that maintains the global climate and the microscopic marine organisms are a huge source of atmospheric oxygen (Landrigan *et al*., 2020). Marine life is a major food source for humans and plays an important role in supporting the economies of many countries in the world (Tubío *et al*., 2021).

1.1.1. Fish

Since people began fishing in the oceans and raising fish for food through aquaculture, aquatic food products have been a staple of the human diet (Tacon & Metian, 2013). It helps improve humans' nutrition, health, and general well-being. As malnutrition is one of the major problems, fish can play a vital part in one's diet because it provides affordable access to essential nutrients such as vitamins, minerals, omega-3 fatty acids, essential amino acids, and trace elements (Maulu *et al*., 2021).

Fish fulfills about 17.1% of the global requirement for good-quality animal protein (Tacon *et al*., 2020) and it provides 75% of the animal protein consumed daily in the majority of emerging nations (Maulu *et al*., 2021). Fish is the primary source of protein in certain island nations, such as Bangladesh and Indonesia (Rehman *et al*., 2019). Globally, there is a high demand for seafood and annual production of fish is increasing. According to the Food and agriculture organization (FAO), the total fish production was more than 178 million tons in 2020, of which about 97 million tons were from captured fish (Ahuja *et al*., 2020).

1.1.2. Aquaculture industry

Aquaculture is described as the farming of the species present in the water such as shellfish or finfish by individuals or organizations by adopting measures like feeding, isolating, medicating, and controlled breeding to increase production (Sapkota *et al*., 2008). According to the FAO, aquaculture has become the fastest-growing food production industry, producing 50% of the aquatic food consumed by humans (Yilmaz *et al*., 2022). Globally, 60-880 million people depend entirely or partially on

fisheries and aquaculture for their livelihood. According to the literature based on current utilization, to satisfy world demand, aquaculture production must rise from 82,087 kilotons in 2018 to 129,000 kilotons in 2050. Aquaculture will predominate the world's seafood supply by 2050 (Xu *et al*., 2022). In addition to being a significant source of revenue, aquaculture also helps many nations with their social development and food security.

Pakistan has a lot of potential for fishing with a 1,120 km long coastline and inland water of 3,102,408 acres which is located in the northern Arabian Sea. In Pakistan's coastal region, about 16,000 fishing boats are used for both shallow ocean waters and offshore locations. Since 1999, Pakistan's inland water aquaculture industry has expanded by 7,230 million tons annually (Shahzad, 2022). Although Pakistan's aquaculture industry is growing, its output growth rate is rather slow when compared to that of other aquaculture-producing countries. It can be increased by enhancing fishing techniques as well as by controlling the death of the fish which is caused due bacterial and other infectious diseases.

1.2. Infectious diseases of fish

Aquaculture increases fish production which can satisfy the growing human population's need for protein. However, the issue of fish infections in aquaculture has become more prominent (Moreira *et al*., 2021). Diseases that have been caused by pathogens which include viruses, bacteria, helminths, and fungi, and outside factors such as poor water quality, poor breeding techniques, and environmental deterioration can severely reduce the yield of fish produced on farms (Ji *et al*., 2020).

In fish farms, diseases by pathogens cause roughly 45% of the damage. Bacterial diseases cause significant loss in both farmed and wild fish (Kousar *et al*., 2019). Vibriosis, winter ulcer, photo bacteriosis, furunculosis, marine flexibacteriosis, pseudomonadiasis, streptococcosis, and bacterial kidney disease are some main bacterial diseases of fish that have caused major losses in the aquaculture industry (Toranzo *et al*., 2005).

It is estimated that ten percent of all aquatic animals raised for food die from infectious diseases each year, costing the industry more than $10 billion in losses worldwide (Adams, 2019). So, for the long-term sustainability of aquaculture, along

with food safety and environmental protection, there is a need to protect the fish from these diseases (Su *et al*., 2021).

1.3. Fish vibriosis

Vibriosis is an infection caused by bacteria belonging to the genus *Vibrio* (Jun & Woo, 2003). It is one of the earliest known marine fish infections called as “red sore” or “red spot” due to its characteristic of causing hemorrhagic skin lesions (Nurliyana *et al*., 2019). The disease was first identified and described in Italy in 1718 (Colwell & Grimes, 1984) and its first confirmed case is reported in 1893. Later, in 1909, one of the main causative agents of vibriosis, *Vibrio anguillarum* was isolated (Emmy, 1987). *V. anguillarum*, *V. vulnificus*, *V. ordalii*, and *V. alginolyticus* have been found to cause fish and shellfish infections more frequently (Sanches-Fernandes *et al*., 2022).

1.3.1. Prevalence of vibriosis

Vibriosis is one of the most widespread bacterial infections which affect a variety of marine fish. 50% of the mortality in fish is caused by vibriosis (Ina-Salwany *et al*., 2019). It is seen as a serious issue with large financial losses in the aquaculture business globally. The shrimp farming business had been severely impacted by this disease, with an economic loss of $1 billion (Nurliyana *et al*., 2019). Aquaculture mortality caused by *V. anguillarum* has been particularly severe, with some instances showing losses of up to 100% (Hickey & Lee, 2018).

1.3.2. Causes and clinical symptoms of vibriosis

Vibriosis outbreaks in farmed fish are caused by changes in the physicochemical parameters of the water including quality of water, pollution, temperature, and salinity (Manchanayake *et al*., 2023). Temperature more than 15°C causes vibriosis. Overcrowding due to massive fish farming is another major cause of vibriosis (Frans *et al*., 2011).

The symptoms of vibriosis begin with outward changes. If left untreated, the infection could spread throughout the body of the fish and cause death (Ina-Salwany *et al*., 2019). Vibriosis's clinical symptoms were initially noted in Norway and then in the United Kingdom in infected eels caused by *Vibrio anguillarum* (McCarthy, 1976). They include tiredness, anorexia, darkened skin, protrusion of the eyeball, necrosis of the gills, respiratory problems, aberrant swimming motion, ulcerative and

hemorrhagic lesions on the skin, and death (Ransangan & Mustafa, 2009; Istiqomah & Isnansetyo, 2020; Manchanayake *et al*., 2023).

1.3.3. Causative agent-*Vibrio anguillarum*

The main causative agent of vibriosis is *Vibrio anguillarum*, also called *Listonella anguillarum*. It is a comma-shaped, gram-negative rod bacteria. It is facultative anaerobic, non-spore-forming, and polarly flagellated as shown in Figure 1.1. It grows rapidly on the media having 1.5 to 2% NaCl with a temperature of 15 to 30°C(Frans *et al*., 2011; Bekaert *et al*., 2021).

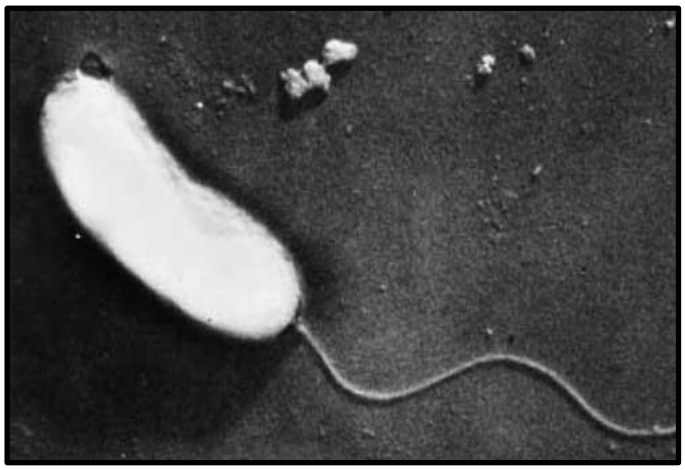

Figure 1.1: Electron micrograph of flagellated *Vibrio anguillarum* (Frans *et al*., 2011)

1.3.4. Mode of transmission of *Vibrio anguillarum*

Contaminated water and food infestation is the main route of transmission of *Vibrio anguillarum* into fish and causes vibriosis. Moreover, the disease transmits through the skin (Weber *et al*., 2010). Although the mucous layer on the skin avoids the attachment of the *Vibrio anguillarum* to the epithelial cells of the fish, the damaged and injurious mucous layer is the main entry point of the bacteria into the fish (Jun & Woo, 2003). After infestation, *Vibrio anguillarum* goes towards the gastrointestinal tract of the fish and proliferates and colonizes in the gut using intestinal epithelium, from which it enters the bloodstream and hence causes sepsis (Frans *et al*., 2011).

1.3.5. Susceptible fish species

More than 50 of the fish species are susceptible to infection by *Vibrio anguillarum*. Most of them are economically important for the aquaculture and larviculture industry such as rainbow trout, eel, salmon, turbot, sea bream, sea bass, cod, and ayu.

Crustaceans and bivalve mollusks are also infected by the *V. anguillarum* (Frans *et al*., 2011).

1.4. Drugs against vibriosis

Antibiotics are used as a treatment for vibriosis in fish aquaculture. But the overuse of these broad-spectrum antibiotics has created resistant bacteria and these resistant bacteria make other bacteria resistant by transferring their resistant genes (Xu *et al*., 2022). So, there is a need of using methods other than antibiotics such as probiotics, chemotherapeutics, vaccines, and other technologies.

Different chemotherapeutics and antibacterial drugs are also used against vibriosis, but severe environmental and health problems are associated with using antibacterial and chemotherapeutics **(**Heenatigala *et al*., 2020; Lee *et al*., 2021). Hence for the management of vibriosis in aquaculture, the development of vaccines is the most effective method.

Vaccines are the agents that induce an immune response for a specific antigen obtained from the pathogen or the infectious agent that causes the disease (Czochor & Turchick, 2014). Different types of vaccines have been developed against fish vibriosis such as live attenuated, inactivated, subunit, and DNA vaccines.

1.4.1. Live attenuated vaccines

The chemically or genetically weakened pathogens are used as vaccines which are called live attenuated vaccines. It creates much immunogenic response as compared to inactivated vaccines but there is a danger in it that the pathogen can be activated due to various reasons (Ji *et al*., 2020).

1.4.2. Inactivated vaccines

Inactivated vaccines are formed using heat or by chemically (formalin) killing the inactivated pathogen. This type of vaccine is not able to reproduce and so needs additional doses. Many such vaccines have been commercially used against fish vibriosis. These include Alpha Marine-vibrio, Norvax Vibriose Marine, AquaVac Vibrio, and MicroViB (Angelidis *et al*., 2006; Frans *et al*., 2011).

1.4.3. Subunit vaccines and DNA vaccines

Subunit vaccines are being considered for the vaccine due to their safety and non-virulence nature as compared to live attenuated and inactivated vaccines. These

vaccines are made by using protein or antigens from the pathogen that functions as the pathogen virulence, movement, attachment, or reproduction are safer and effaceable (Xing *et al*., 2017). Recombinant subunit vaccine is formed by expressing the potential antigen into other microorganisms and DNA vaccines are being developed by encoding the specific antigen into a plasmid and then expressing it in other organisms (Frans *et al*., 2011).

According to the literature, DNA and recombinant subunit vaccines against *V. anguillarum* are being made using flagellins (FlaA and FlaB and outer membrane proteins (OmpK, OmpR, OmpU, and VAA) (Xu *et al*., 2022). A DNA vaccine based on the EmpA (zinc metalloprotease) has been reported to have worked well against *V. anguillarum* in 2009 (Yang *et al*., 2009).

Several vaccines based on the recombinant outer membrane proteins of *V. anguillarum* have demonstrated efficient and long-lasting protection in recent years (Zhou *et al*., 2018; Xing *et al*., 2020; Li *et al*., 2022). In addition, it was shown in the research that bivalent vaccines made of two proteins provide particularly good protection against *V. anguillarum* infection (Zhou *et al*., 2018).

1.4.4. Plant-based vaccines

Recombinant proteins that are expressed in plants are considered the safer form of vaccines. There are different techniques of administration of vaccines such as injection, immersion, and oral route. While considering techniques for giving vaccines to fish, injection is much more laborious and time-consuming. The vaccine can also be administered by immersion, but it is regarded as less immunogenic and need additional doses (Frans *et al*., 2011; Mondal & Thomas, 2022).

Vaccination by the oral route is much simple and cheap as compared to other methods of vaccine delivery for giving the vaccine to a large mass of fish, but the main problem that arises in the oral route of vaccination is that the antigen has to pass down the fish gut where there is a chance that it can be degraded due to acidic environment (Mondal & Thomas, 2022).

This problem can be solved by developing edible plant-based vaccines by expressing the antigens in plants. Plants have thick and rigid cell walls in which the antigen is encapsulated and protected from the acidic environment of fish gut **(**Heenatigala *et al*., 2020). Moreover, plant-based vaccines are cost-effective, safer, stable, and easily

scalable. There is no need for post-translational modification as well (Kim *et al*., 2009; Lössl & Waheed, 2011; Su *et al*., 2021).

1.5. Outer membrane protein K (OmpK)

Outer membrane proteins (OMPs) are present on the outer surface of gram-negative bacteria. It maintains integrity and provides semi-permeability to bacterial outer membranes **(**Huzmi *et al*., 2020**).** As these proteins have a conserved nature and are present on the outer surfaces of bacteria so they can be easily recognized by the immune system of the host and are regarded as highly immunogenic. Hence, they are now the focus to be used as a potential candidate for vaccine development. (Heenatigala *et al*., 2020).

OmpK is a protein that is widely distributed in *Vibrio* species. It functions as a semipermeable outer membrane of *V. anguillarum*, acts as an adhesion molecule, and a receptor. Due to this nature, it is considered a highly immunogenic and potential vaccine candidate against fish vibriosis (Ji *et al*., 2020).

Many studies have demonstrated OmpK as a protective antigen and potential vaccine candidate against vibriosis. In 2012, OmpK from *Vibrio anguillarum* was cloned in *Escherichia coli* to produce a recombinant subunit vaccine against vibriosis which generated high immunogenicity in animal trials (Hamod *et al*., 2012). Different other studies have also shown good relative percent survival (RPS) rate and production of antibodies, and T and B lymphocytes when OmpK from *V. anguillarum* is genetically engineered to make a subunit vaccine against fish vibriosis.

In several studies, researchers have created multivalent subunit vaccinations by combining various immunogenic antigens with OmpK to boost the protective impact of subunit vaccines on bacteria. Innate immune response, cytokines production, and antigen-specific antibodies have been obtained by using a multivalent vaccine containing OmpK and FlaB against fish vibriosis (Lee *et al*., 2021). However, so far, no plant-based expression of OmpK antigen has been reported.

1.6. Plant biotechnology

Plant biotechnology is broadly defined as the use of plants, microorganisms, and living processes for well-being. Old plant biotechnology includes agriculture, and the production of crops and goods (Boulter, 1995). At the start of the 1970s, with the

emergence of the concept of genetic engineering, the foundation of modern biotechnology had been made.

Modern biotechnology is defined as the production of transgenic plants (Sussex, 2008). In 1983, *Agrobacterium*-mediated transformation has been explained by three research groups and hence made the history of modern plant biotechnology (Vasil, 2008). It is used for the improvement of economically important plants with more desirable characteristics, and the production of biopharmaceuticals, vaccines, and antibiotics (Buonaguro & Butler-Ransohoff, 2010).

1.7. Plant tissue culture

Plant tissue culture is the aseptic and *in vitro* production of cells, tissue, organs, and their subcomponents within physical and chemical conditions (Thorpe, 2006). Haberlandt, who is regarded as the father of tissue culture, performed experiments on single-cell culture and was the first who explained tissue culture on a theoretical basis (Thorpe, 2007). His experiments made the foundation of the principle of totipotency of cells (García-Gonzáles *et al*., 2010). Proper tissue culture conditions along with basal media provide all necessary nutrients, water, and energy for a plant or explant to grow into a new plant.

Tissue culture is used in large-scale production and multiplication of plants. It is a main research tool. Moreover, it has many industrial applications in the field of propagation of plants, production of secondary metabolites, elimination of plant diseases, and formation of improved crops (Hussain *et al*., 2012).

1.7.1. Explants

Explants are the small parts taken from the plants that are capable of regenerating into a new entire plant. Explants can be part of the leaves, stems, roots, or flowers of plants (Idowu *et al*., 2009). In tissue culture, we use different types of explants i.e., cotyledonary nodes, or hypocotyl parts as shown in Figure 1.2, and provide them with optimized environment conditions along with the proper concentration of nutrients and hormones.

The Choice of the type of explant, size, age, excise position, orientation, and hormone treatment is very important while performing pant transformation (Chakraborty *et al*., 2020). By providing controlled environmental and nutrient conditions, these explants

can produce a new plant irrespective of the external weather and environmental conditions (Hussain *et al*., 2012).

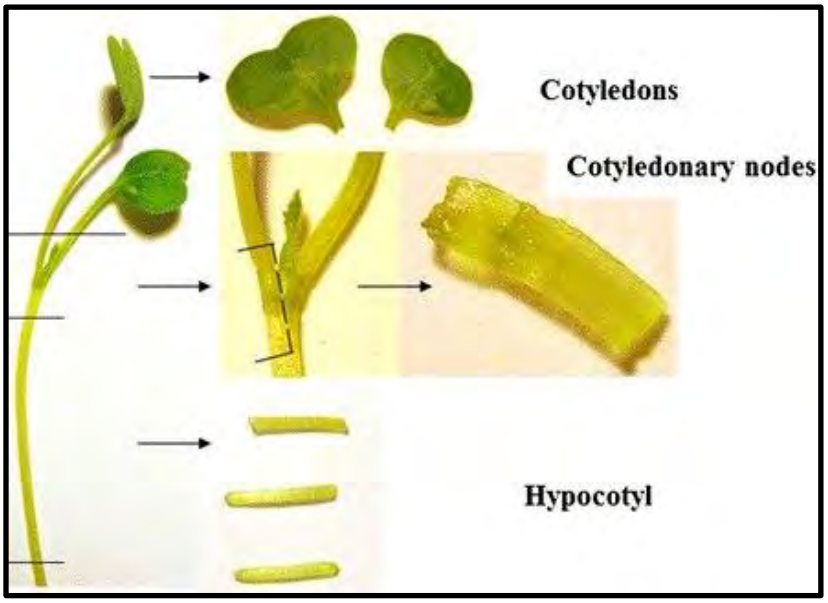

Figure 1.2: Types of explants used for the plant tissue culture (modified from Qi *et al*., 2014)

1.7.2. Culture media of plant

Along with the optimized light, incubating environment, and temperature condition, for the tissue culture, plants need media for growth from which they can get necessary nutrients, energy, and water called basal media. Different types of basal media have been used for this purpose (Phillips & Garda, 2019). Many attempts have been made for the nutrient medium that allows isolated cells and tissues of plants to grow. In the United States, White was the first scientist who was able to grow plant cells for an indefinite period. He named the medium as White's medium (White, 1934). Another graduate from the laboratory of Folke Skoog, Murashige got a fourfold increase in growth than White, by adding large amounts of nitrate, ammonium, phosphate, and potassium salts in White's medium (Vasil, 2008). This results in a new and defined medium called Murashige and Skoog medium or MS medium (Murashige & Skoog, 1962). Chelated iron, myoinositol, and four vitamins have also been added to it. MS media is now the most widely used medium for plant tissue culture (Vasil, 2008). There are other media available too for the regeneration of plants i.e., BABI or B5, Schenk and Hildebrandt medium (SH medium), WPM, and DKW (Phillips & Garda, 2019).

1.7.3. Growth regulators of plant

Plant growth regulators also known as phytohormones are synthetic as well as natural substances (Gaspar *et al*., 1996). They have their role in the growth and development of plants. They have specific effects based on the genotype of the plant and the type of explant. They are used in low amounts and are used in combination with two or more hormones (Evans *et al*., 1981). These compounds are used in plant tissue culture.

There are five main classes of naturally present phytohormones: Auxins, Cytokinins, Gibberellins, abscisic acid, and ethylene (Phillips & Garda, 2019). Auxins and cytokinins and their interaction are the most important in the tissue culture settings and these two classes are majorly used in the regeneration of plant tissues. Other classes have only regulatory roles and are mostly ignored in the cultures (Rademacher, 2015).

Some synthetic compounds are also present which behave like natural plant hormones, for example, synthetic Auxins i.e., 2,4-D (2,4-dichlorophenoxyacetic acid) and picloran (Phillips & Garda, 2019). These are more potent than the natural ones. There are some of them which act like inhibitors, and they are used to study the natural phytohormones in *in vitro* studies (Davies, 1995).

1.7.3.1. Auxins

Auxins are a group of compounds that are chemically diverse and mostly contain aromatic rings in their structure such as indole, naphthalene, or phenyl ring along with a carboxylic group attached to the side chain (Bajguz & Piotrowska, 2009). Auxins are involved in callus formation, the extension of roots and shoots as well as somatic embryogenesis (Zhang *et al*., 2022). They perform cell elongation and promote cell division. In combination with cytokinins, they promote differentiation of the xylem and phloem (Cooke *et al*., 2002; Jamil *et al*., 2021). The first plant growth regulator which is isolated is indole-3-acetic acid (IAA). Other examples of auxins are indole butyric acid (IBA), and naphthalene-1-acetic acid (NAA) (Phillips & Garda, 2019).

1.7.3.2. Cytokinins

Cytokinins are phytohormones that are N^6-substituted derivatives of adenine with an aromatic or isoprenoid side chain (Bajguz & Piotrowska, 2009). They are involved in all the phases of plant development (Riefler *et al*., 2006).

They promote cell division which helps in shoot regeneration, the development of apical shoots, and the formation of undifferentiated calluses (Phillips & Garda, 2019). High concentrations of cytokinins inhibit root formation. Kinetin was the first identified cytokinin. Zeatin and 2-ip (2-isopropyl adenine) and zeatin are the two other naturally occurring cytokinin used in the tissue culture. BAP (6-benzyl amino purine) and thidiazarun are synthetic cytokinins that are used commonly in tissue culture (Gaba, 2005).

1.8. Plant biopharming

Biopharming is described as the production of biological therapeutics outside the living body using living systems as hosts. These living systems are used as bio-factories to produce biological materials which are economically or technically not possible with the other *in-vitro* systems (LeBlanc *et al*., 2020). The first incident of this method was Genentech's use of *Escherichia coli* as the bacterial host to generate insulin in 1978 and it is commercialized later in 1982 (Quianzon & Cheikh, 2012). Afterward, eukaryotic cells are also employed as hosts, for example, the use of CHO (Chinese hamster ovary) cells to produce anticoagulants (Jayapal *et al*., 2007) and monoclonal antibodies (Reinhart *et al*., 2015). Nowadays, biopharming is being employed using yeast, mammalian, avian, insect, plant, and bacterial expression systems (Legastelois *et al*., 2017).

The use of plants as hosts to produce biological materials has advantages over other systems. Unlike fermenter-based systems, which need much time and cost, plant-based systems are cost-effective and flexible for the amplification of biomass (LeBlanc *et al*., 2020). The first example of biopharming in plants is the development of chimeric human growth hormone using sunflower and tobacco (Barta *et al*., 1986). Many different other plants have been transformed such as tobacco, maize, lettuce, barley, tomato, rice, broccoli, pitcher plants, carrot, and soybean (Kim *et al*., 2012; Miguel *et al*., 2019).

1.9. Plant-based edible vaccines

About 54% of the total mortality in developing countries is caused by infectious diseases and the most suitable way of protection from infectious diseases is through vaccines (Concha *et al*., 2017). Conventional methods of vaccine development such as fermenter-based systems are less scalable and costly. Most of the vaccines are

expensive due to their costly manufacturing and distribution process. According to WHO, there is a need for cost-effective vaccines in developing countries. In this regard, Plant-based vaccines are cost-effective and have many other advantages over other systems (Lössl & Waheed, 2011). Plant-based vaccines are made by the expression of genes of disease-specific antigens into plant hosts using genetic engineering methods. It can be done by direct bombardment of genes into plants or by using *Agrobacterium*-mediated transformation (Kurup, 2020). The idea of plant-based vaccines gathered attention due to the reason that vaccines can be edible. The edible vaccine is those which can be ingested orally and elicit an immune response (Langridge, 2000).

Using edible plants as hosts, specific antigens can be expressed by different genetic engineering techniques. After ingesting an edible vaccine, the antigens are prevented from stomach secretion breakdown due to the rigid cell wall of plant cells. Hence the antigens are delivered to the mucosal surfaces of the intestine, where they get absorbed by various processes to trigger a potent and localized immune response (Pelosi *et al*., 2011). The method of producing an edible plant-based vaccine that elicits an immune response is demonstrated in Figure 1.3.

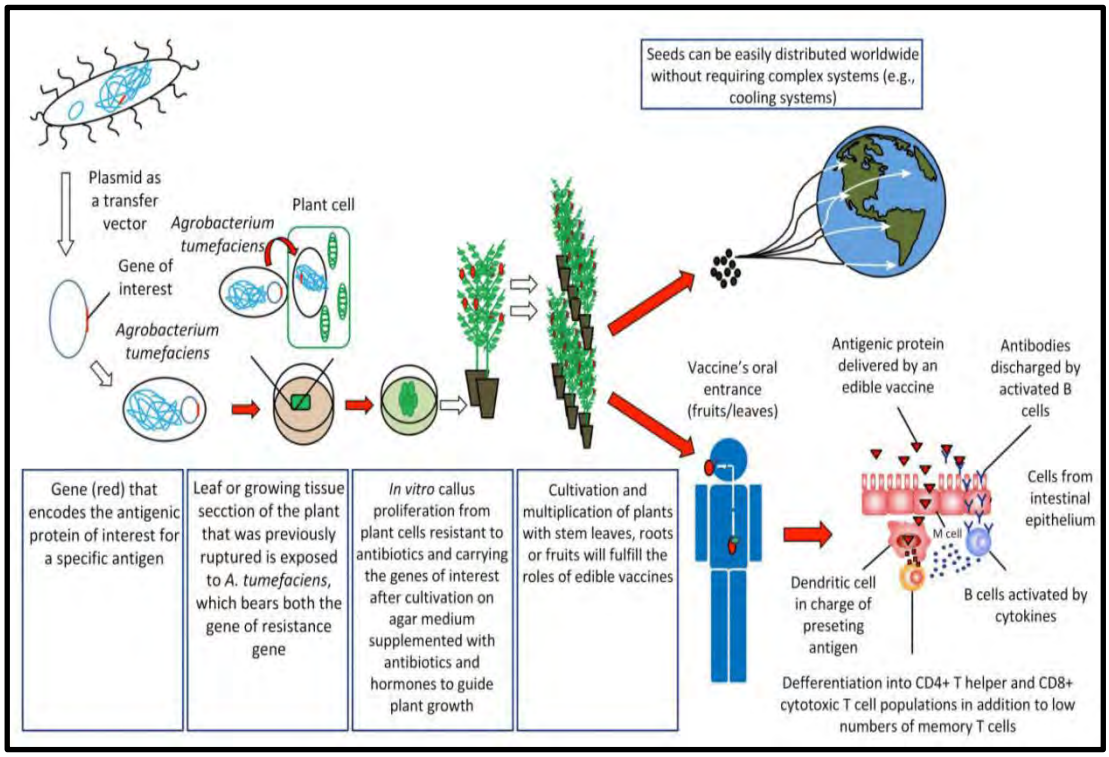

Figure 1.3: Method of producing plant-based edible vaccines and its immune action (Concha *et al*., 2017)

1.9.1. Advantages of plant-based edible vaccines

The major advantage of using plant-based systems is their cost-effectiveness. Plants can be cultivated on the site where it is required, and they can be scaled up according to need as compared to costly instruments and infrastructure of time-consuming fermenter-based systems (Waheed *et al*., 2016). Due to the stability of antigens in plant systems, there is no cost for the maintenance of the cooling chain, specific transportation, and storage. There is no chance of contamination due to pyrogens or endotoxins. So, there is no need for stringent purification and downstream processing as well (Lössl & Waheed, 2011). They can be administered orally in raw form. Moreover, they are covered with a rigid cell wall which makes them protective against the gut's acidic environment (Sack *et al*., 2015). According to the literature, there is proper folding of antigens in plant systems, and more immunogenic responses and increased expression have been demonstrated in plant-based systems (Waheed *et al*., 2015).

1.9.2. Disadvantages of plant-based edible vaccines

Some risks associated with plant-based vaccines are that they may cause allergic reactions, hypertensive responses, or oral tolerance (Takeyama *et al*., 2015). Proper dosage optimization is necessary in this case. There is a chance of incorrect post-translational modifications. Another issue associated with plant-based expression is that they may contaminate non-transgenic plants present in the field (Malabadi *et al*., 2015). However, this issue can be resolved by the expression of antigens in chloroplasts, because there is no chance of transgene dispersal via pollen (Waheed *et al*., 2016).

1.10. Plant transformation

Plant transformation means the integration of the foreign DNA into the plant's cells and then the regeneration of these transgenic plants. It began in the early 1980s when it was demonstrated that the *Agrobacterium tumefaciens* which causes the grown gall disease in plants can be manipulated by researchers to integrate foreign genetic material into plants (Newell, 2000). The first attempt at plant transformation is done on a tobacco plant in 1984 (Hansen & Wright, 1999)

There are two methods of transformation i.e., direct, and indirect methods. In the direct methods, genetic materials are introduced via non-biological systems using

physical and chemical methods. In indirect methods, biological systems such as bacterial cells are used to introduce foreign genes into plant cells (Rakoczy-Trojanowska, 2002) as demonstrated in Figure 1.4. The different direct methods available include biolistic or particle bombardment by gene gun, silicon carbide fiber mediated gene transfer, polyethylene glycol method (PEG method), laser microbeam method, microinjection, lipofection, and sonication method. Direct methods include *Agrobacterium*-mediated gene transfer, agroinfiltration, and viral vector-based transformation (Keshavareddy *et al*., 2018).

Either stable or transient expression of the foreign DNA is possible because of plant transformation (Newell, 2000).

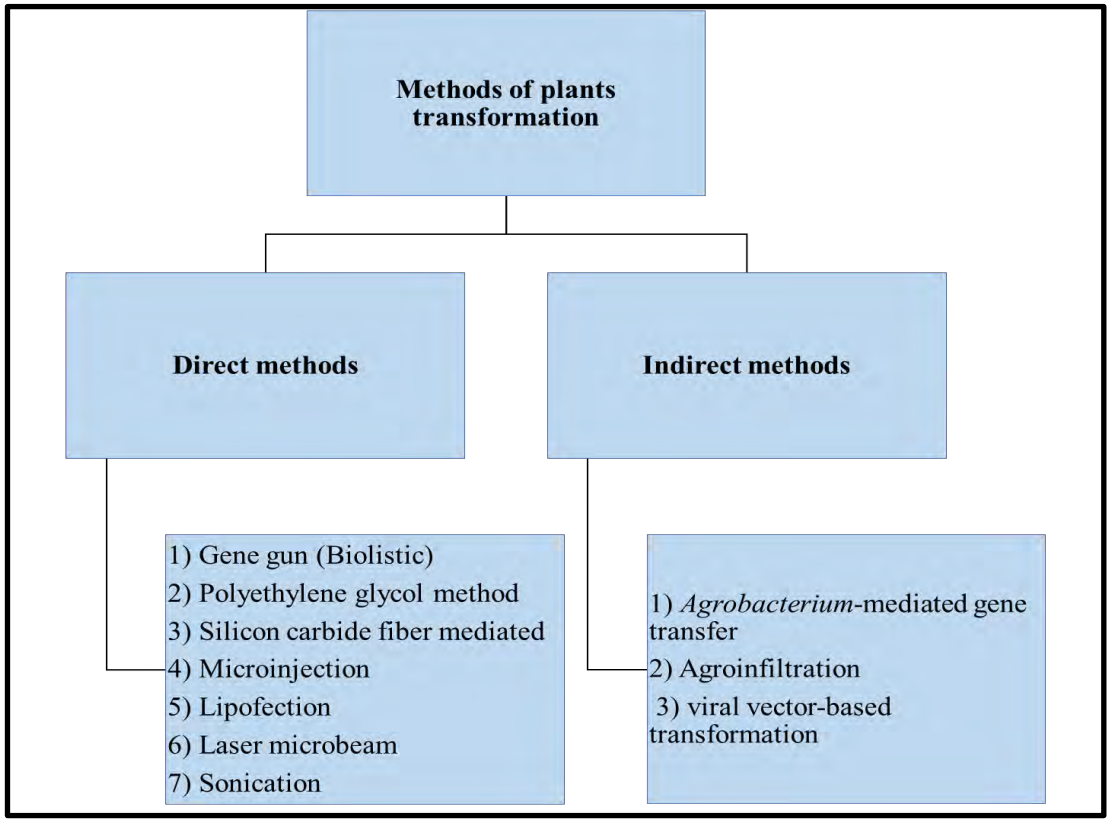

Figure 1.4: Methods of plant transformation (modified from Saifi *et al*., 2020)

1.10.1. *Agrobacterium*-mediated transformation

Agrobacterium is considered a powerful tool for transferring the gene of interest into the host plant cells and it is considered as an efficient means of transferring foreign genes into the plants (Matveeva, 2018). *Agrobacterium* has the natural ability to transform host plant cells by introducing its T-DNA present in its tumor-inducing (T_i) plasmid into the plant cell nucleus (Chilton *et al*., 1977).

The transferred T-DNA naturally contains tumor-inducing genes and opines metabolic genes which when transferred into plant cell induces uncontrolled growth of the transformed cells and the opines or amino acids derivatives produced are used by the bacteria to meet its nitrogen needs (Tzfira & Citovsky, 2006).

The transformation can be stable or transient. Inside the nucleus, the transferred DNA may integrate into the host plant cell genome and transmit into the next generation called stable transformation or it can transiently transform the plant cell by settling in the nucleus and doesn't integrate into the host genome called a transient transformation (Hwang *et al*., 2017).

1.10.1.1. Stable transformation

For the stable nuclear transformation of foreign genes in plants, *Agrobacterium* is commonly used. As a result of this, foreign genes are integrated into the plant cell nucleus and transferred to the next generations in a stable manner (Horn *et al*., 2004).

1.10.1.1.1. Advantages and disadvantages of stable nuclear transformation

Advantages:

The main advantages of stable *Agrobacterium* nuclear transformation are that it has high efficiency in the transformation of plant cells, and it provides the constant expression of the protein product of the gene of interest (Laere *et al*., 2016). The method is cost-effective as compared to other methods (Saba *et al*., 2019).

Disadvantages:

The disadvantage of stable nuclear *Agrobacterium* transformation is that it is a time-consuming method as compared to transient transformation (Waheed *et al*., 2016). Moreover, random integration may result in the positional effect. Gene silencing and transgene contamination may occur as a result of stable nuclear transformation (Fahad *et al*., 2015).

1.10.1.1.2. *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a gram-negative soil-borne bacteria. It is a phytopathogen that causes crown gall disease in many plants (Gelvin, 2010). Crown gall is a plant tumor and *Agrobacterium* has this tumor-inducing ability because of the presence of a tumor-inducing (T_i) plasmid. T_i has a DNA called transferred DNA (T-

DNA) which integrates into the host plant cell nucleus and induces crown gall or tumor (Niazian *et al*., 2017).

Figure 1.5 shows the structure of *Agrobacterium tumefaciens*. Genetic research on *Agrobacterium* over the past 100 years has changed the molecular genetics of plants and produced a brand-new business devoted to genetically modifying plants.

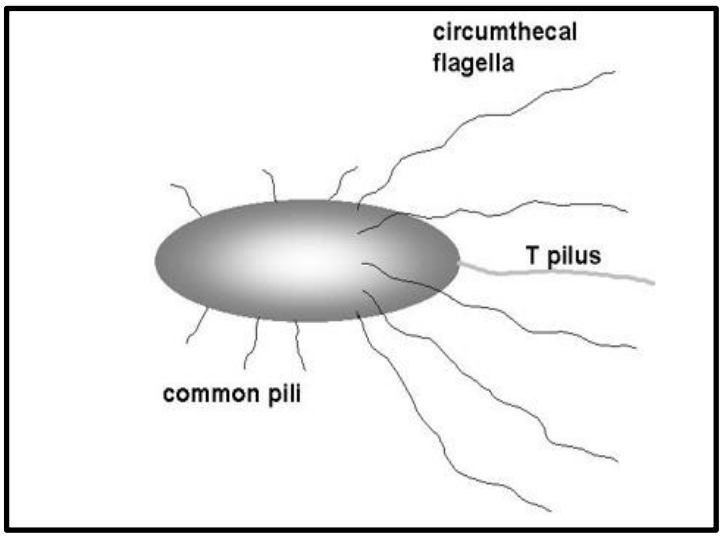

Figure 1.5: Structure of *Agrobacterium tumefaciens* (Ali *et al*., 2010)

1.10.1.1.3. T_i plasmid

T_i plasmid of *Agrobacterium* contains two important genetic components which are required in the *Agrobacterium*-mediated transformation process. These two components are T-DNA and virulence (*vir*) region (Ziemienowicz, 2014). *Vir* region contains genes that encode bacterial virulent proteins. These proteins help in T-DNA processing and its delivery into host plant cells (Hwang *et al*., 2017). It also has opine catabolism genes and the origin of the replication region. The diagrammatic representation of T_i plasmid is shown in Figure 1.6.

1.10.1.1.4. T-DNA

The T-DNA carries oncogenic genes, and these genes encode different enzymes. These enzymes synthesize cytokinins and auxins which cause tumor formation in plants (Barampuram & Zhang, 2011). T-DNA also contains opine formation genes that satisfy nitrogen needs. T-DNA is flanked by two repeats of 25-bp called the right border and the left border as shown in Figure 1.6. These repeats help in the transfer of T-DNA by acting as a *cis*-element signal (Zupan *et al*., 2000).

It is evident from tumor formation that the T-DNA integrates and expresses in the plant cells. So, any foreign DNA or gene of interest that can be placed within the T-DNA borders can be transmitted to the plant cells. This makes the base of *Agrobacterium*-mediated transformation (Alimohammadi & Bagherieh-Najjar, 2009).

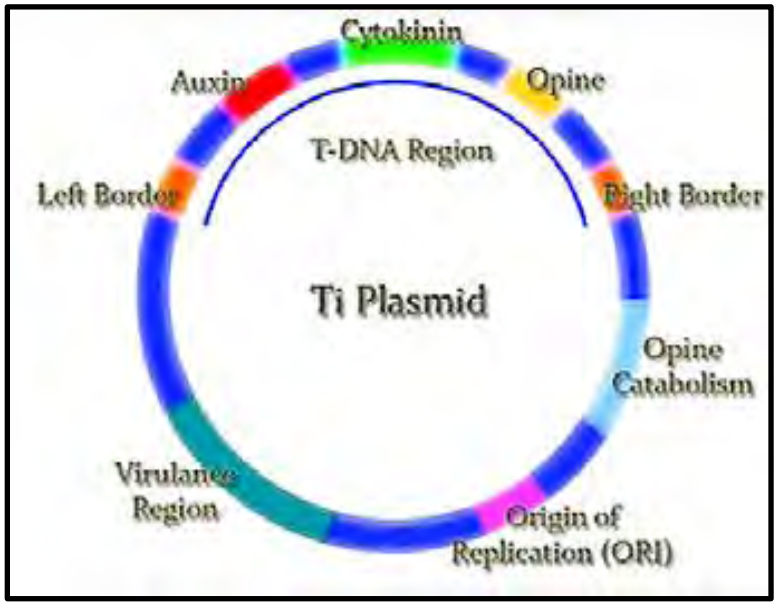

Figure 1.6: Diagrammatic representation of T_i plasmid (Alkuddsi *et al*., 2014)

1.10.1.1.5. Steps of T-DNA integration for stable transformation

The delivery of T-DNA inside the host cell genome comprises different steps. 1) Release of signals by plant cells 2) Activation of *vir* gene expression 3) T-DNA synthesis and processing 4) T-DNA and vir proteins transport into plant cell 5) T-DNA and vir protein complex importation into plant cell nucleus 6) T-DNA complex integration and expression (Pitzschke & Hirt, 2010) as demonstrated in Figure 1.7.

Plant cells signaling and recognition by *Agrobacterium*

The process starts when *Agrobacterium* receives signals from the plants. These include phenols, sugars, low pH, and low phosphate signals (Brencic & Winans 2005). As bacteria perceive these signals, expression of some *vir* genes is induced (Pitzschke & Hirt, 2010). The most effective signals include monocyclic phenols such as acetosyringone (Stachel *et al*., 1985). Some of the studies have reported that this process starts from the wounded site of the plant where *Agrobacterium* infects plants at the wounded site. Along with the point of entry, the wounded site of plants releases an acidic sap containing high content of phenolic compounds which helps in the

chemotaxis of bacteria (Barampuram & Zhang, 2011). On sensing of these compounds by bacteria, *vir* genes expression is activated.

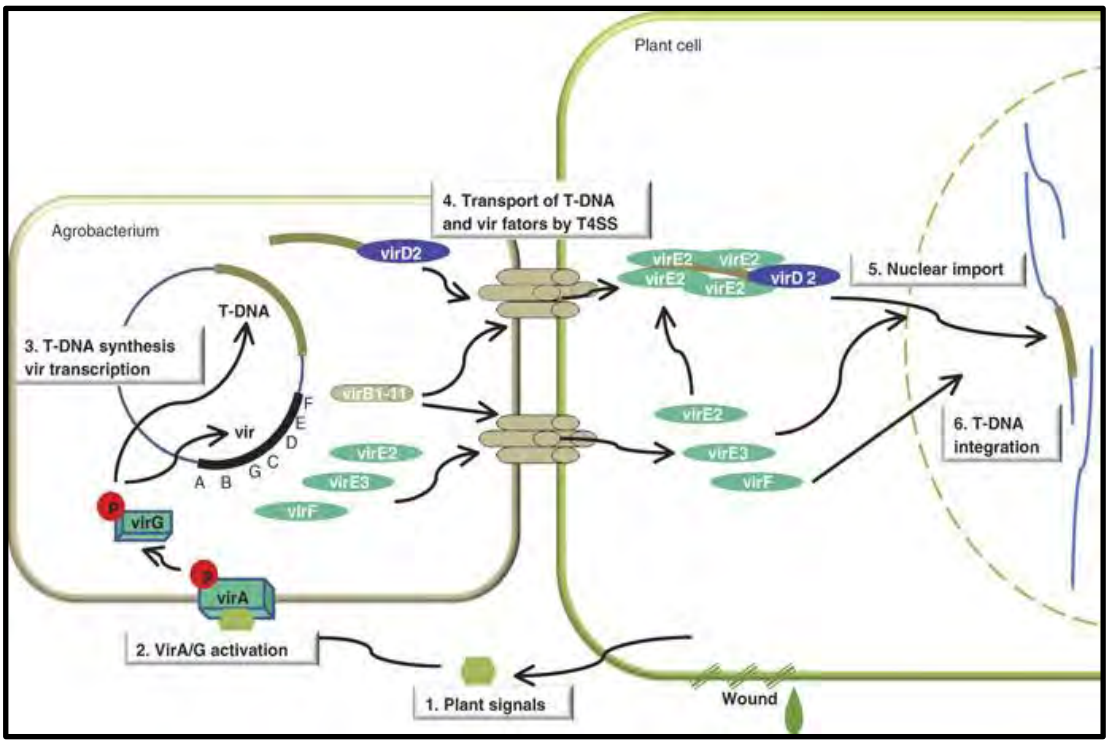

Figure 1.7: Overview of the bacteria and plant interaction (Pitzschke & Hirt, 2010)

Activation of *Agrobacterium vir* gene expression

The expression of two *vir* genes (*vir A* and *vir G*) is enhanced by signals released from plant cells. These genes are expressed constitutively at a basal rate (Pitzschke & Hirt, 2010). Vir A is a membrane-bound sensory protein, and it is activated first on getting the signals by plant cells, and it activates another vir G by phosphorylating its aspartate residue. Vir G acts as an intracellular response regulator (Wolanin *et al*., 2002) and binds to DNA segments of *vir* gene promoters and activates transcription. Thus, it helps in activating other vir proteins (Brencic & Winans, 2005).

T-DNA synthesis and processing

Vir gene expression results in the formation of single-stranded T-DNA which is also called T-strand. VirD1 and virD2 proteins are involved in this process. They bind the left and right borders of the bottom strand of T-DNA and cleave the supercoiled T-DNA into a single stand (T-strand) which is bonded with the virD2 at its 5' on the right border side (Hwang *et al*., 2017). This single strand then enters the host cell via other vir protein. Before entering, the T-strand is conjugated with virD2 and gets

coated by virE2 forming the T-complex. It functions to protect the T-strand from degrading inside the host plant cell (Barampuram & Zhang, 2011).

T-complex transport into host plant cell cytoplasm

The T-complex is transported into the host plant cell cytoplasm by the type-4 secretion system. Vir B protein complex (B1-12) and virD4 make the T4SS complex as shown in Figure 1.8 and this complex is involved in this transportation. T-complex leaves the bacteria and enters the host cell cytoplasm via T4SS and is then transported to the nucleus of the host cell (Christie, 1997).

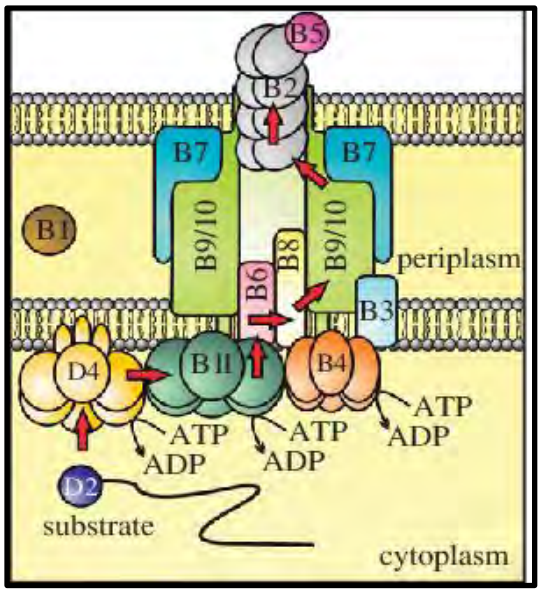

Figure 1.8: Type-4 secretion system (T4SS complex) comprising virD4 and virB (Zechner *et al*., 2012)

T-DNA transport into the nucleus and integration into the host genome

For the transport of T-complex from the cytoplasm to the nucleus, various bacterial vir proteins and plant proteins are involved. Both virD2 protein which is covalently bonded to T-complex and virE2 to which T-complex is coated contain nuclear localization signal (NLS) that guides the T-strand into the nucleus of the host cell (Rossi *et al*., 1993; Ziemienowicz *et al*., 1999).

Inside the host cell nucleus, the T-complex is degraded by virF and proteasomal machinery of the host cell. As a result, T-DNA is released from the complex and its integration into the host genome is facilitated (Tzfira *et al*., 2004).

Integration of T-DNA inside the genome of host plant cells is random, where expression of transgene occurs, and the process is called stable transformation (Gelvin, 2017).

The precise mechanism of T-DNA integration into the plant genome and the role that specific proteins play in this process are not fully understood. Some studies have shown that VIP2, a protein that interacts with VirE2, has been shown to be crucial for T-DNA integration into plant genomes (Anand *et al*., 2007). T-DNA integrates into plant chromosomes using a similar method to how foreign DNA mostly integrates into plants through recombination or non-homologous end-joining (Pitzschke & Hirt, 2010). The whole process of T-DNA nuclear transport and integration is demonstrated in Figure 1.9.

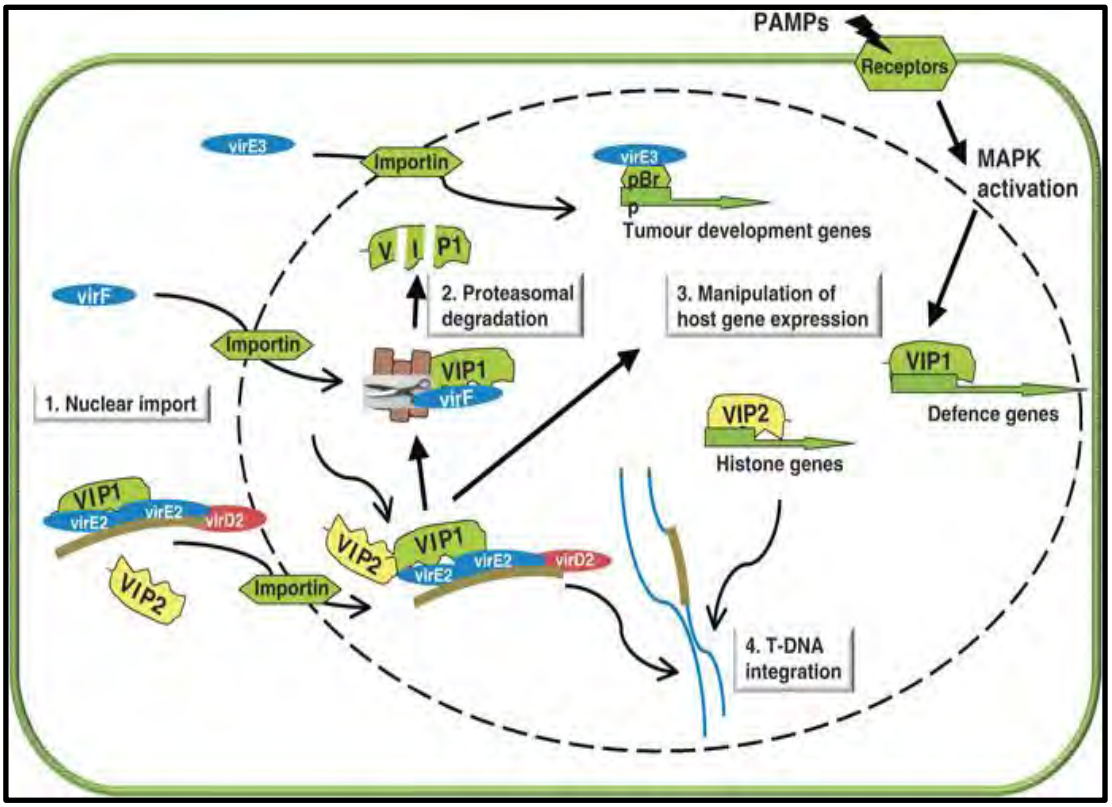

Figure 1.9: T-DNA nuclear transport and integration (Pitzschke & Hirt, 2010)

1.10.1.1.6. Mechanism of stable transformation

The stable *Agrobacterium*-mediated transformation consists of various steps which are demonstrated in Figure 1.10. The steps include 1) Transformation of *Agrobacterium* with the gene of interest 2) Seed sterilization, Inoculation, and preparation of bacterial suspension 3) Preparation of the explants, Infection, and co-

cultivation of plant and *Agrobacterium* 4) Washing and transfer into selection plates 4) callus formation 5) Shoot regeneration 6) Elongation and rooting 7) Acclimatization of the transformed plant (Collado *et al*., 2015).

Various factors influence the success rate of stable *Agrobacterium*-mediated transformation. These include the cultivar of the plant, type of the explant use, density of bacterial suspension, time of infection, co-cultivation time, the correct concentration of acetosyringone, and type and concentration of selection antibiotic (Asande *et al*., 2020).

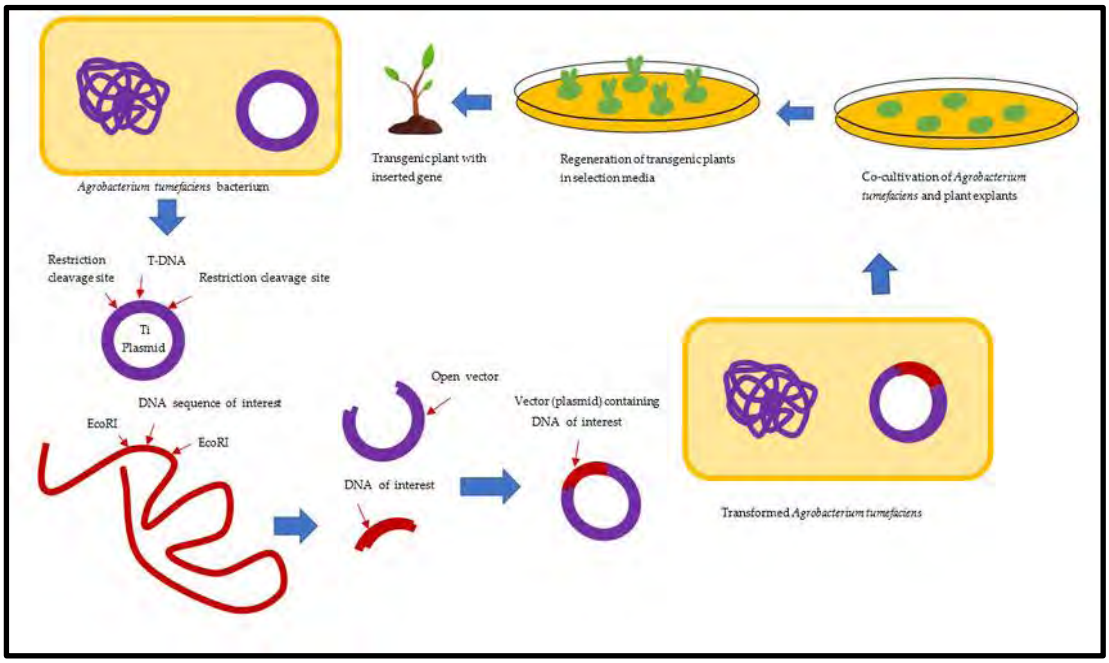

Figure 1.10: Mechanism of *Agrobacterium*-mediated transformation (Ghimire *et al*., 2023)

1.10.2. Transient plant transformation

Transient expression of genes in plants does not involve the integration of the transgene in the plant genome and hence does not follow the central dogma expression pattern. But it gives a way to express foreign recombinant proteins in very less time (Fahad *et al*., 2015; Tyurin *et al*., 2020).

Advantages:

The main advantage of the transient expression is that it is rapid and flexible (Fischer *et al*., 1999) and the recombinant protein can be attained within some days. Moreover,

its transformation efficiency is more as compared to stable transformation and it does not require laborious tissue culture techniques (Kaur *et al*., 2021).

Disadvantages:

The disadvantage is that it is not suitable for the large-scale production of recombinant protein. Its expression has a short life, and the transgene is lost when the replication of the host cell occurs (Fischer *et al*., 2012).

Different methods can be used for the transient transformation of the gene of interest i.e., biolistic method, polyethylene glycol (PEG) mediated transformation, electroporation, and agroinfiltration using *Agrobacterium* (Wang & Jiang, 2011; Tsuda *et al*., 2012; Tan *et al*., 2013).

1.10.2.1. Agroinfiltration

Agroinfiltration is a simple, quick, and efficient way to introduce the desired gene into the host cell (Norkunas *et al*., 2018). It involves the physical or vacuum infiltration of transformed *Agrobacterium* carrying the target gene directly into the extracellular leaf spaces (Donini & Marusic, 2019) as shown in Figure 1.11.

Single-stranded T-DNA from *Agrobacterium* was transferred to plant cells through agroinfiltration and then transported to the nucleus by chaperones. As a result, a large amount of short-lived recombinant protein production occurs by the expression of T-DNAs that do not integrate into the plant cell genome (Voinnet *et al*., 2003). Agroinfiltration provides a high level of transformation efficiency, great scalability, and simplicity for many transient expressions using several transgenic vectors and genes containing the desired gene on a single leaf (Shoji *et al*., 2008).

Syringe agroinfiltration, the most widely used technique for agroinfiltration, involves injecting *Agrobacterium* into plant leaves using a needleless syringe (Santi *et al*., 2008). First, a tiny nick is made in the epidermis of the leaf's backside with a needle by lightly scratching without damaging. Then, using a needleless syringe, *Agrobacterium* from the infiltration medium is injected into the leaf through the nick. The light green tint of the leaf starts to darken as the *Agrobacterium* combination infiltrates the intercellular space of leaf, showing successful and effective infiltration (Chen *et al*., 2013). The whole protocol of agroinfiltration is demonstrated in Figure 1.11.

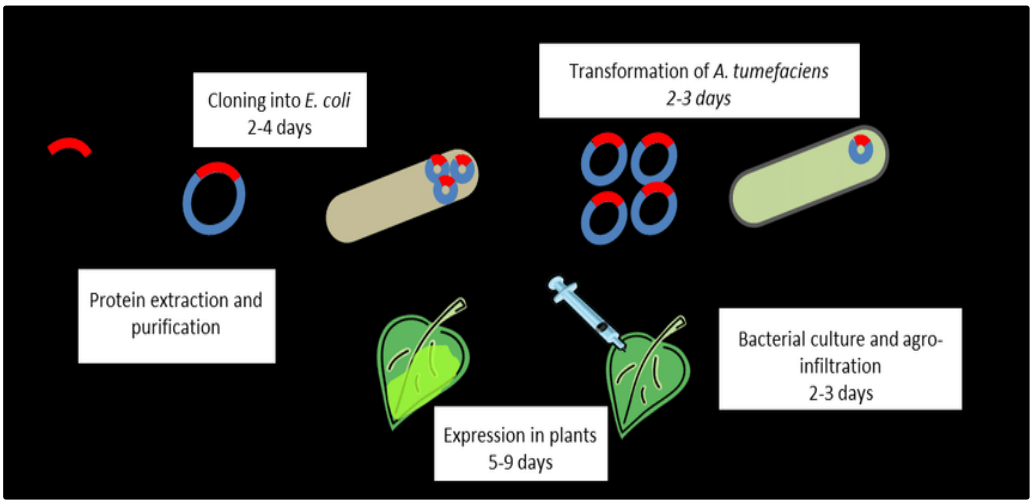

Figure 1.11: Agroinfiltration protocol using a syringe (Peyret, 2014)

1.10.3. Gene gun-mediated transformation

Gene gun (biolistic) or particle bombardment method is another method of the transformation of plant cells (Matsumoto & Gonsalves, 2012). In this method, DNA or gene of interest is directly bombarded on the plant cells using a gene gun. The DNA is first coated to metal, usually gold or tungsten particles, and then bombarded on the plant at high speed (Twyman & Christou, 2004). High pressure is maintained by using helium gas. As a result, DNA penetrates the plant cell and successfully delivers the gene of interest to the plant cell (Ozyigit & Yucebilgili Kurtoglu, 2020).

1.11. *Solanum lycopersicum* L.

Solanum lycopersicum L. (Tomato) is one of the most significant fruits or vegetables which has a high nutritional value and is grown throughout the world (Sunilkumar *et al*., 2016). It belongs to the Solanaceae family and botanically it is classified as (a berry) fruit (Tripathy & Mallikarjunarao, 2020; Krishna *et al*., 2022). It is diploid having 24 chromosomes (2n) (Causse *et al*., 2020). The origin of tomato is South America (Peru), and it is first domesticated in Mexico (Sharma *et al*., 2019). Tomatoes were discovered to be used in cooking by the Aztecs in Mexico as early as 500 BC, and after the conquer of Aztec territory, tomatoes were introduced to the rest of the world (Bergougnoux, 2014).

Tomatoes are grown in various varieties with different colors and shapes all over the world. According to the latest FAO report, the United States, India, and China are the

major countries that grow tomatoes (Toni *et al*., 2021). Tomato is consumed in raw form or used in different dishes, sauces, drinks, and salads (Ray *et al*., 2016).

The tomato is known by various other names throughout the world. It was generally referred to as the love apple (Jenkins, 1948). It is known as tomate in German, jitomate in Spanish, garden tomato, or just tomato, in English, and tamater in Urdu (Waheed *et al*., 2020).

Tomatoes and the products made by them are full of nutritious products which are good for health. It prevents chronic diseases and cancer due to the presence of antioxidants in it (Lana & Tijskens, 2006). Tomatoes are a rich source of amino acids and vitamins (A and C), and they have low calories (Ray *et al*., 2016; Toni *et al*., 2021). The tomato contains several biologically active substances, including lycopene, β-carotene, kaempferol, quercetin, tocopherol, luteolin, and apigenin (Alam *et al*., 2019; Toni *et al*., 2021).

1.11.1. Taxonomic classification

Tomatoes are placed in the *Solanaceae* family. This family contains more than 3000 species and many of them are economically important crops including potatoes, peppers, eggplant, petunias, and tobacco (Bai & Lindhout, 2007).

The tomato was given the scientific name *Solanum lycopersicum* L. by Carolus Linnaeus in 1753 (lyco is Latin for "wolf," and persicum is Spanish for "peach"). However, in 1754, Philip Miller separated the tomato and several other species into a new genus, Lycopersicon, and gave the tomato the name *Lycopersicon esculentum* Mill. (esculentum = "edible"). Miller may have wished to point out that the fruit of tomato was edible because at the time many people believed it to be harmful (Knapp & Peralta, 2016). According to the International Code of Botanical Nomenclature (ICBN), the name *Lycopersicum* should be used instead of Lycopersicon esculentum. Nevertheless, until recently, *Lycopersicon esculentum* was frequently utilized (Kimura & Sinha, 2008). The complete taxonomic classification of *Solanum lycopersicum* L. is given in Table 1.1.

The genus *Solanum* is a member of the *Solanaceae* plant family, one of the most significant to humans and it is one of the major angiosperm genera. There are thought to be 1500 species in this genus. There are currently 13 recognized species of wild tomatoes included in this genus e.g., *Solanum chmiewelskii*, *Solanum chilense*,

Solanum pennilli, *Solanum neorickii*, *Solanum habrochaites*, *Solanum ochranthum*, *Solanum juglandifolium*, *Solanum sitiens*, *Solanum lycopersicoides*, *Solanum arranum*, *Solanume corneliomuelleri*, and *Solanum galapagense* (Peralta *et al*., 2006; Kimura & Sinha, 2008).

Table 1.1: Taxonomic classification of *Solanum lycopersicum* L. (Krishna *et al*., 2022)

Kingdom	Plantae
Phylum	Angiosperms
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	*Solanum*
Species	*Lycopersicum*

1.11.2. Morphology

The tomato is a perennial plant that grows to a height of about 3m. Its leaves are compound in most genotypes, pinnate, and alternate with 5-9 tiny leaflets on the petiole. Its stem is fragile and hairy in early stages; but as it ages, it becomes hard, woody, and densely branched (Sharma *et al*., 2019).

Tomato possesses a tap root system and has a highly branched network of fibrous and adventitious roots (Cheema & Dhaliwal, 2004). Tomatoes are mostly self-pollinated crops, but in exceptional instances, 30% cross-pollination has been seen (Rai *et al*., 2013).

Solanum lycopersicum L. has perfectly formed, hypogynous flowers that are carried on a short pedicle with a cyme inflorescence which is monochasial or dichotomous branched. Flowers are generally yellow and are bisexual. The stamens, which are made up of an anther carried on a filament, represent the male portion of the flower. A flask-shaped cone with a protruding sterile tip is formed by the anthers joining

laterally. The pistil, which consists of the stigma, style, and ovary, is the female reproductive organ of the tomato flower. It is found in the core of the flower and is surrounded by stamens (Krishna *et al*., 2022).

Berry fruits have many light brown or golden yellow seeds, and when ripe, these seeds seem to be orange, yellow, or red (Lawal *et al*., 2007). The fruit of the tomato varies greatly in size and shape, ranging from round and small to big or maybe having other diverse shapes. They are circular, oval, elongated, flattened, or heart-shaped (Krishna *et al*., 2022). The fruits also vary in color. They are found in red, purple, green, or yellow color (Atherton & Rudich, 2012). Most varieties of tomatoes contain two or more locules (Bhatnagar-Mathur *et al*., 2007). Figure 1.12 shows all morphological features of the tomato plant i.e., seeds, leaves, flowers, fruits, seedling, and completely grown tomato plant.

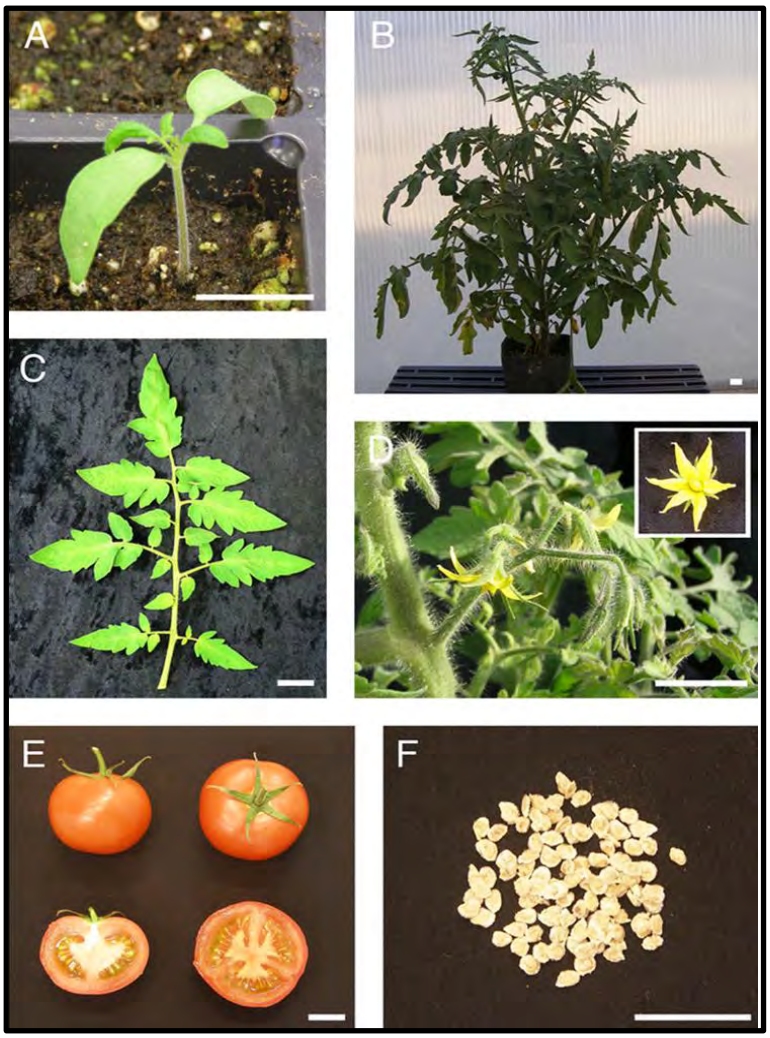

Figure 1.12: Morphology of tomato plant (A) seedling (B) 1-month-old tomato plant (C) leaves (D) flowers (E) fruit shape and color (F) seeds (Kimura & Sinha, 2008)

Tomatoes can be grown in a variety of soil types, including clay, black, and red soil, although sandy loam soil that is rich in organic matter is best. The tomato can live in moderately acidic and saline soils. Its cultivation is reported to be best at a temperature of 15–27°C; however, at daytime temperatures more than 38°C may negatively impact fruit set (Krishna *et al*., 2022).

1.11.3. Use in biotechnology

The tomato is a good candidate for a model plant species due to its diploidy, self-fertility, reasonably short life cycle, a large array of germplasm resources, reasonably compact sequenced genome (950 Mb) and its genetic linkage map (Pujar *et al*., 2013; Gerszberg *et al*., 2015; Alsamir *et al*., 2021).

It can be used as an alternative to *Arabidopsis thaliana*, which is commonly used as a model plant due to the range of germplasm resources and specific plant characteristics like light period, ability of seed production, flowering, production of compound leaves, fleshy fruits, and mycorrhizal roots (Carvalho *et al*., 2011; Gerszberg *et al*., 2015).

Other advantageous qualities of tomato to be used as a model plant are broad spectrum of tomato mutations, lack of gene duplication, homozygosity, and ability of asexual reproduction (Alsamir *et al*., 2021; Gerszberg *et al*., 2015).

Existing tomato cultivars can be easily employed in genetic research (Sun *et al*., 2006). Moreover, Tomatoes can be produced successfully under different conditions of cultivation and in greenhouses with light durations of 8 to 16 hours, temperatures ranging from 10 to 35°C, and a moisture content of 30 to 90% which makes it suitable for being used as a model plant (Schwarz *et al*., 2014).

Most of the techniques like protein extraction, RNA isolation, and genomic DNA extraction protocols devised for Arabidopsis can be easily applied to tomatoes as well. Tomatoes can be used in tissue culture method to stably transform the gene of interest (Kimura & Sinha, 2008).

In 1986, the first *Agrobacterium*-mediated transformation of tomatoes was reported. Since then, multiple transformation techniques involving various explants, such as hypocotyls, cotyledons, leaves, and fruits, have been devised for various tomato cultivars (McCormick *et al*., 1986; Wu *et al*., 2011).

1.12. Aims and objectives

Following were the aims and objectives of the study.

- To optimize the concentration of hygromycin for nodal explants of *Solanum lycopersicum* L. for the identification of transformed plants
- Stable *Agrobacterium*-mediated transformation of *Solanum lycopersicum* L. with *OmpK* gene for the development of plant-based vaccine against fish vibriosis
- To confirm the transformation by performing different molecular biology techniques such as PCR and qRT-PCR
- To estimate and quantify the OmpK protein by Dot blotting, Western blotting, and ELISA

MATERIALS AND METHODS

This study aimed to optimize the concentration of hygromycin for the selection of transformed plant using *Solanum lycopersicum* L. plant, to optimize the best conditions for the transformation of *Solanum lycopersicum* L., and to develop an efficient stable transformation protocol for *Solanum lycopersicum* L. cv. Rio Grande with *OmpK* gene via *Agrobacterium*-mediated transformation. Moreover, the study also focused on the optimization of Polymerase Chain Reaction (PCR) conditions for the *OmpK* gene and the confirmation of the integration and expression of *OmpK* gene in transformed explants using different molecular biology techniques. In this section, a detailed methodology that was followed along with the apparatus used in this study will be explained. The present research work was done in the Plant Biotechnology Lab, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

2.1. Materials

2.1.1. Laboratory instruments

In this study, different types of instruments were used made by different manufacturers. These were used by following proper guidelines for each instrument. The details of all these instruments are given in the following Table 2.1.

Table 2.1: Laboratory instruments

Appliances	**Company**
Autoclave	Yamato, USA
Balance	Ohaus Corp, USA
Blotting apparatus	Major Science, USA
Centrifuge	Eppendorf, Germany
Colibri microvolume spectrophotometer	Titertek Berthold, Germany
Conventional PCR	Applied Biosystems, USA

Electroporator	Biorad, USA
Freezer	Dawlance, Pakistan
Freezer (-70°C)	VWR, USA
Gel casting device	Cleaver Scientific, USA
Gel documentation system	Alpha View SA Version 3.4.0.0, USA
Magnetic stirrer	VWR, USA
Minitron incubator	VWR, USA
Micropipettes	Eppendorf, Germany
Orbital shaker	VWR, USA
Power supply	Biometra, USA
Real-Time PCR	Mygo Pro, Ireland
Spectrophotometer/Microplate reader	ThermoScientific multiskan GO
Vertical Gel electrophoresis apparatus	Cleaver Scientific, USA
Vortex	Scientific Industries, USA
Water Bath	Precision, 180 Series, USA

2.1.2. Chemicals and reagents

High-purity chemicals and reagents were used in this research work. They were purchased from different suppliers and manufacturers. The solutions of chemicals and reagents were made using distilled water as a solvent. The details of chemicals and reagents are summarized in Table 2.2.

Table 2.2: Chemicals and reagents

Chemicals	**Company**
Agar (Bacteriological grade)	Bioworld, Dublin, Germany
Agar (Plant TC)	Phytotech lab, US
Ammonium phosphate (APS)	AnalaR^{TM}, England
Ammonium sulfate($(NH_4)_2SO_4$)	GPR, BDH laboratory supplies, UK
Ascorbic acid	AnalaR^{TM}, England
Bovine Serum Albumin (BSA)	MP Biomedical, USA
Boric acid	AnalaR^{TM}, England
Bromophenol Blue	Sigma-Aldrich, USA
β-Mercaptoethanol	Biochem, USA
Calcium Chloride	AnalaR^{TM}, England
Cefotaxime	Sanofi-aventis, Pakistan
Chloroform ($CHCL_3$)	Sigma-Aldrich, USA
Coomassie	AnalaR^{TM}, England
CTAB	Oxford lab chem, India
Dimethyl sulfoxide (DMSO)	Biochem, USA
Absolute Ethanol 99.7-100% pure v/v	Merck, Germany
Ethidium Bromide	Sigma-Aldrich, USA
Ethylenediamine Tetra acetic acid (EDTA)	AnalaR^{TM}, England

Glacial Acetic acid	Merck, Germany
Glucose	GlaxoSmithKline, UK
Glycerol	AnalaR^{TM}, England
Glycine	Merck, Germany
HEPES, Free Acid ($C_8H_{18}N_2O_4S$)	Phytotechnology Laboratories, USA
Hydrochloric acid	Sigma-Aldrich, USA
Imidazole	Sigma-Aldrich, USA
Isoamyl-alcohol	AnalaR^{TM}, England
LB Broth (Miller)	Microgen, India
Magnesium chloride 6-hydrate ($MgCl_2.6H_2O$)	AnalaR^{TM}, England
Mercuric chloride ($HgCl_{2)}$	AnalaR^{TM}, England
Methanol	Sigma-Aldrich, USA
Phenol	Merck, Germany
Potassium Chloride (KCl)	AnalaR^{TM}, England
Propanol-2 (Propa-2-ol)	AnalaR^{TM}, England
SDS (Sodium dodecyl Sulfate)	Phytotechnology Laboratories, USA
Sodium acetate (CH_3COONa)	AnalaR^{TM}, England
Sodium Chloride (NaCl)	Applichem, USA
Sodium Hydroxide Pellets	Merck, Germany
Sodium Metabisulphite	Sigma-Aldrich, USA

Sucrose	AnalaR^{TM}, England
Sulfuric acid	Sigma-Aldrich, USA
Tris-base	Invitrogen, USA
Tris-(hydroxymethyl) aminomethane	Phytotechnology Laboratories, USA
Triton X-100	AnalaR^{TM}, England
Tween-20	Sigma-Aldrich, USA

2.1.3. Ready-to-use kits and reagents

In this experimental work, high-quality, cost-effective, and ready-to-use kits and reagents were used. The details of these kits and reagents are given in Table 2.3.

Table 2.3: Ready-to-use kits and reagents

Reagents	**Company**
Anti-His antibody	Abcam, USA
Chemiluminescence system	ECL® PLUS; GE Healthcare
Complete protease inhibitor	Sigma-Aldrich, USA
dNTP set	Thermofisher, USA
Horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG	Abcam, USA
1kb DNA Ladder	Thermofisher, USA
4-10% NuPAGE® Bis-Tris Gel	Thermofisher, USA
Ni-NTA agarose	Thermofisher, USA
Horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG	Abcam, USA

Protein Ladder	Thermofisher, USA
Proteinase K	Thermofisher, USA
Nitrocellulose membrane	Thermofisher, USA
RNase A	Bioworld
SYBR green	Thermofisher, USA
3,3,5,5-tetramethylbenzidine (TMB)	Thermofisher, USA
Taq DNA Polymerase (5U/µl)	Thermofisher, USA

2.1.4. Laboratory glass and plastic wares

Different plastic wares and glassware were used in this research work. Glass wares were reused in this experimental work after being washed with commercial liquid detergent, dipped in bleach for some hours or overnight, and dried in the dry oven at 150°C. After drying, they were autoclaved for sterilization by maintaining the 15psi (pound per square inch) pressure at 120°C for 25 minutes. The details of all these consumables are given in Table 2.4.

Table 2.4: Consumables

Consumables	**Company**
Glassware	Pyrex, USA
1.5/2 ml Eppendorf tubes	Axygen Scientific, USA
15/50 ml Falcon tubes	Corning® USA
Gloves	Qualtex Malaysia
Jars	Pakistan
Microtiter plates	Costar, USA
Nitrile gloves	Gen-X, Malaysia

Parafilm PM-96	Bemis, USA
PCR tubes	Thermo Scientific USA
Petri plates	Pyrex, USA
Pipette Tips	Extra Gene, USA
Surgical blades	XINDA, China
Syringe filters (0.2 µm)	Sartorius, Germany
Whatman filter paper	GE Healthcare UK

2.1.5. Primers

In this study, OmpK-nuclear forward, *OmpK*-nuclear reverse, *OmpK* internal forward, and *OmpK* internal reverse primers were used. The sequences of these primers are given in Table 2.5.

Table 2.5: Primers

Primers	**Sequences**
OmpK-nuclear forward primer	**5'**GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGTAAATCACTTTTAGCTCTAGGCC**3'**
OmpK-nuclear reverse primer	**5'**GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG**3'**
OmpK internal forward	**5'**CTAAGCAACCCAAGCAGTGACAAAG**3'**
OmpK internal reverse primer	**5'**CCTTCATCTTTCAGACCATAAACATCTTTGTAGC**3'**

2.2. Laboratory working precautions

Sterile conditions are required for the work of plant tissue culture and transformation. Sterile conditions must be maintained to avoid contamination and cross-contamination. The following actions were taken to achieve the purpose.

- The lab coat was worn while doing all the work in the laboratory.
- A pair of gloves was worn while working with plants and chemicals.
- All the apparatus and media used in the lab work were autoclaved for 20 minutes, at 121°C with maintained pressure of 15 psi.
- All the working areas and benches were surface sterilized with 70% ethanol.
- Laminar Flow Hood (LFH) was used, and the surface was sterilized by swabbing with 70% ethanol and followed by exposing it to ultraviolet (UV) radiation for 20-30 minutes.
- All the equipment to be used in the lab work (except chemicals, antibiotics, hormones, bacterial cultures, seeds, and plants) was also surface sterilized with 70% ethanol and kept under UV radiations within LFH.
- Hands were also sterilized by spraying with 70% ethanol before working in the sterile LFH.
- After working, hands were cleaned with soap or sanitizer.
- Contaminants were treated with strong bleach and autoclaved before being discarded.

2.3. Sterilization of Laminar Flow Hood (LFH)

Sterile working conditions were ensured by the LFH which has a High Efficiency Particulate Absorption (HEPA) filter. The LFH was properly swabbed with 70% ethanol (or spirit) before use. UV was switched on for 20 to 30 minutes to maintain thorough surface sterilization. Before being placed in the LFH, the equipment needed for the work within the hood was sprayed with 70% ethanol (alternative: spirit), and it was also subjected to UV rays for 20–30 minutes. UV was turned off after 30 minutes, and hands were then sprayed with 70% ethanol (or spirit) before being brought into the sterile environment of LFH. To sterilize tools like forceps and scalpels before use, they were placed in 70% ethanol and heated over a flame until red-hot.

2.4. Culture media preparation

We used different media in this research work for different purposes. For seed germination, we used Murashige and Skoog (MS) media, and different strengths of

MS medium were used with optimized plant growth regulators (PGRs) for the regeneration of plants. For bacterial inoculations, Luria Broth (LB) media was used, and Luria Agar (LA) media was used for streaking the bacterial inoculum.

2.4.1. Murashige and Skoog (MS) media

We have used different strengths of MS media for seed germination and plant regeneration. For seed germination, we used ½ MS (Annexure 2.2), and for tomato plant regeneration, we used full MS with vitamins (Annexure 2.2).

For the preparation of media, at first, calculated amounts of sucrose and MS (basal salts for seed germination and MS with vitamins for plant regeneration) were dissolved in distilled water. The solution's pH was measured using a pH meter, and it was kept between 5.75 and 5.85 by using 0.1N (Normal) NaOH. The calculated amount of phyto-agar was added once the pH had been adjusted. After being tightly closed, the reaction bottle was autoclaved for 20 minutes at 121°C and 15 psi. Under sterile LFH conditions, the medium was put into many jars (each holding 50 ml) and in the small petri plates (each holding 25ml). The jars were used for seed germination and the petri plates were used for the explant's regeneration. The jars and the Petri plates were sealed with parafilm and kept in the growth room.

2.4.2. Bacterial culture media

2.4.2.1. Luria Broth (LB) media

LB media was used to inoculate bacteria. LB media was made in a pre-autoclaved reagent bottle and the calculated amount of LB (Annexure 2.2) was dissolved in the required amount of distilled water. The media was autoclaved at standard conditions and was stored at 25°C and later, used for the inoculation of bacteria with the calculated amount of antibiotics added for the selection.

2.4.2.2. Luria Agar (LA) media

Streaking of bacterial inoculum was done in LA media (Annexure 2.2). The media was prepared by dissolving the calculated amount of LB and bacterial agar (Annexure 2.2) into the required amount of distilled water in a pre-autoclaved reagent bottle. The media was autoclaved and cooled down slightly.

Calculated amounts of antibiotics were added for bacterial selection within LFH conditions. For the present study, 50mg/l kanamycin and 50mg/l rifampicin were

added from the stock solutions (Table 2.6). Finally, it was poured into Petri plates (25ml) which were then sealed using parafilm. The wrapped petri plates were stored in the growth room and were used for the streaking later.

2.5. Preparation of stock solutions

In this research work, stocks of different plant hormones and antibiotics were used in different concentrations. Their compositions are described in Table 2.6.

Table 2.6: Stock concentrations of different hormones, antibiotics, and chemicals

S.No.		Stock solutions	Concentration
1.	Hormones	Zeatin	2mg/ml
2.	Antibiotics	Hygromycin	50mg/ml
		Kanamycin	50mg/ml
		Rifampicin	2mg/ml
		Cefotaxime	250mg/ml
3.	Chemicals	Acetosyringone	100mM
		Mercuric chloride ($HgCl_2$)	0.1%

2.5.1. Preparation of stock solutions of plant hormones

Stocks of plant hormones were prepared which were used for plant regeneration.

2.5.1.1. Zeatin

2mg/ml stock of zeatin was prepared in autoclaved distilled water. 200mg powder of zeatin was weighed and added to 10 ml of autoclave distilled water in a falcon within the LFH chamber. The power was allowed to mix in the distilled water by stirring. After complete dissolution, the stock was filter-sterilized using a membrane filter with 0.2 µm filter pore size and was stored at -20°C.

2.5.2. Preparation of stock solutions for antibiotics

Different working concentrations of antibiotics were used in this study. Methods of the preparation of the stocks of all the antibiotics which were used in the research work are as follows.

2.5.2.1. Hygromycin

50mg/ml stock was prepared by dissolving 500mg of solid hygromycin into 5ml sterile distilled water within LFH. Upon complete dissolution, the volume was raised to 10ml with distilled water. The stock was filtered and sterilized using a membrane filter with 0.2 µm filter pore size and was stored in small aliquots at -20°C.

2.5.2.2. Kanamycin

50mg/ml stock solution of kanamycin was prepared by dissolving 500mg powdered kanamycin in 5ml of autoclaved distilled water within the LFH chamber. When the solution became clear, the volume was raised to 10ml. The stock was filtered and sterilized using the filter assembly and was stored in small aliquots at -20°C.

2.5.2.3. Rifampicin

The stock solution of rifampicin was prepared in Dimethyl Sulfoxide (DMSO) as rifampicin is insoluble in distilled water. 20mg/ml stock was prepared by dissolving 200mg rifampicin in 5ml DMSO within the LFH chamber. After the solid component has been completely dissolved, the volume was raised to 10ml, and the solution was filter sterilized using a membrane filter with 0.2 µm filter pore size. The stock was then stored at -20°C in small aliquots for future use.

2.5.2.4. Cefotaxime

250mg/ml stock was prepared by dissolving 1g cefotaxime in 4ml autoclaved distilled water within the LFH chamber. After the powder has been completely dissolved, the solution was filter sterilized using a membrane filter with 0.2 µm filter pore size. The stock was then stored at -20°C in small aliquots for future use.

2.5.3. Chemicals

Stocks of the chemicals that were prepared in the study are as follows.

2.5.3.1. 0.1% Mercuric chloride ($HgCl_2$)

0.1% Mercuric chloride ($HgCl_2$) was used for the seed sterilization. To make 0.1% $HgCl_2$, 0.05g of mercuric chloride powder was weighed in a paper. The powder was then dissolved in the 50ml autoclaved distilled water within the LFH chamber in a 50ml falcon tube. The powder was allowed to dissolve completely and then stored at 4°C for further use.

2.5.3.2. Acetosyringone

The stock was made in Dimethyl Sulfoxide (DMSO). 100mM stock of acetosyringone was prepared. For this purpose, 196mg of acetosyringone was weighed and dissolved in 10ml DMSO within the LFH chamber. After the dissolution of the powder, the solution was filter sterilized using a membrane filter with 0.2 µm filter pore size. The stock was then stored at -20°C in small aliquots for future use.

2.6. Germination of seed

2.6.1. Plant material

Tomato seeds cv. Rio Grande were used in the study. The seeds were brought from the Awan Seed Shop in Islamabad, Pakistan. The seeds were kept dry and stored at room temperature with a 30% relative humidity in 15ml falcons that were parafilm-sealed.

2.6.2. Seeds sterilization

Seeds were surface sterilized before inoculating them on the germination media. They were sterilized within the sterile conditions of LFH. This procedure was carried out to decrease the likelihood of bacterial and fungal contamination, which would otherwise affect seed germination. Tomato seeds were surface sterilized using 0.1% mercuric chloride ($HgCl_2$). The seeds were treated with 0.1% $HgCl_2$ for 15-20 seconds with constant stirring in a falcon tube so that every side of the seed gets in contact with the chemical. After treatment, the seeds were washed three times with autoclaved distilled water for 3 minutes each and then dried on a sterile filter paper. The seed sterilization steps for tomato seeds are summarized in Table 2.7.

Table 2.7: Seed sterilization steps of tomato

Steps	Method	Solutions	Time
1.	Surface sterilization	0.1% $HgCl_2$	15-20 seconds
2.	3 times washing	Distilled water	3 minutes each

2.6.3. Tomato seed germination media

½ MS and full MS (Annexure 2.2) was used for the germination of sterilized tomato seeds. The media was prepared, autoclaved, and poured into sterile jars. Seeds were

inoculated in these jars. The jars were sealed with parafilm and were placed in the Growth room at 25 ºC in 16/8 hours light/dark cycle.

2.6.4. Tomato seed germination efficiency

After sterilizing tomato seeds, the seed germination efficiency was calculated. Three batches of tomato seeds were tested separately, with a two-week interval between each batch. The following formula was used for seed germination efficiency:

Seed germination efficiency (%) = (Number of seeds germinated) / (Total number of seeds inoculated) × 100

2.7. Preparation of tomato explants

A small fragment of a node was used to regenerate the plant by using tissue culture. The nodes were excised from the grown plant and placed in the regeneration media. Nodal explants were tissue cultured for regeneration, antibiotic optimization, and transformation.

2.7.1. Nodal explants preparation

Nodal explants were prepared by using a 2-3 weeks-old, *in vitro*-grown tomato plant with the help of sterile forceps, and a scalpel with a blade. At first, leaves were excised from nodes and then the nodes were cut. 1.5-2 cm long pieces of nodal explants were used for plant regeneration, antibiotic optimization, and transformation purpose.

2.7.2. Regeneration of tomato explants

For the regeneration of nodal explants of tomato, we used optimized regeneration media i.e., full MS media with vitamins (Annexure 2.2) along with a calculated concentration of zeatin. The regeneration media for tomato nodal explants is given in Table 2.8.

Table 2.8: Regeneration media for tomato plant

S.No.	Components	Composition
1.	MS Strength	Full MS with vitamins
2.	Hormone (Zeatin)	2mg/l

2.7.3. Regeneration efficiency of tomato nodal explants

To determine the efficiency of the regeneration medium for tomato nodal explants, nodal explants were grown on the regeneration medium described above. This experimental work was performed in three different batches. Every second day for 30 days, visual observation was conducted to assess the growth efficiency of nodal explants. The following formula was used to determine the shoot regeneration efficiency of tomato nodal explants:

Shoot regeneration efficiency (%) = (Number of shoots regenerated) / (Total number of explants inoculated) × 100

2.8. Optimization of Hygromycin for tomato nodal explants

Hygromycin optimization was done to determine the appropriate amount of hygromycin needed for transformation and the selection of transgenic explants. Five different concentrations of hygromycin were used i.e., 0, 10, 15, 20, and 25 mg/l. Optimization was done in triplicates and each batch had 5 nodal explants. These were kept in the growth room and were observed for 3-4 weeks.

2.9. Media preparation for transformation

GV3101 strain of *Agrobacterium*, which contains the pGWB5 binary vector, was used to transform *Solanum lycopersicum* L. var. Rio Grande. The transformation of nodes was done by using three different types of media comprising full MS with optimized hormone and antibiotic concentrations.

2.9.1. Co-cultivation media preparation

Co-cultivation media (Annexure 2.3) for nodal explants was prepared by adding acetosyringone and zeatin (optimized concentrations) to full MS media. The media was then poured into sterile petri plates within the LFH. After the media was set, the plates were sealed using parafilm and were stored in the growth room until further use.

2.9.2. Washing media preparation

For the washing media, liquid ½ MS (Annexure 2.2) was used. After maintaining the pH, the media was autoclaved and cooled down before use. Cefotaxime (500mg/l) was added to it before use.

2.9.3. Selection media preparation

Selection media (Annexure 2.3) was prepared by adding hygromycin (25mg/l), cefotaxime (250mg/l), and zeatin to full MS media (Annexure 2.1). The media was poured into sterile petri plates and sealed using parafilm. The prepared selection plates were then stored in the growth room for further use.

2.10. First transformation- taking gene of interest into *Agrobacterium tumefaciens* strain

In this bacterial transformation, *Agrobacterium tumefaciens* were transformed with binary vector pGWB5 containing our target gene, *OmpK.*

2.10.1. Preparation of bacterial culture

In this research study, two bacterial strains, DH5-α, and GV3101 *Agrobacterium* were used. LA plates with 50mg/l kanamycin for selection were streaked with glycerol culture of pGWB5 vector transformed in DH5-α. The streaked plates were kept in the incubator at 37°C for 24-48 hours until the bacterial colonies grow up. A single colony was picked with an autoclaved and sterile loop and was inoculated in 50ml LB (Annexure 2.2) with the calculated concentration of kanamycin (50mg/l). The inoculated LB was then kept in an incubator shaker for optimal bacterial growth. When turbidity was observed in the inoculated media, OD at 600nm was measured using a spectrophotometer (ThermoScientific multiskan GO). As the OD_{600} value of 0.6-0.8 was attained, the flask was removed from the shaking incubator and placed at 4ºC to halt further bacterial growth. We have used different working concentrations of different antibiotics for bacterial selection and transformation, and these are given in Table 2.9.

Table 2.9: Working concentrations of different antibiotics used for the bacterial growth and selection of transformed bacteria

Antibiotics	Working concentration	Stocks prepared
Ampicillin	100mg/L	100mg/L
Kanamycin	50mg/L	50mg/L
Rifampicin	50mg/L	20mg/L

2.10.2. Transformation with *Agrobacterium tumefaciens*

Electrocompetent cells of *Agrobacterium tumefaciens* were transformed with binary vector pGWB5 containing our gene of interest i.e., *OmpK.*

2.10.2.1. Preparation of electrocompetent cells

For this research work, we prepared electrocompetent cells of the GV3101 *Agrobacterium* strain. Electrocompetent cells were prepared by shifting the bacterial inoculum in LB media into a 50ml falcon. It was centrifuged at 2400 rpm for 20 minutes at 4°C and the pellet was dissolved in 40ml chilled distilled water. Again, it was centrifuged at 2400rpm for 20 minutes at 4°C and the pellet was dissolved in 20ml chilled distilled water. Centrifugation was done again, and the pellet was dissolved in 10ml chilled distilled water. Finally, it was centrifuged again, and the pellet was dissolved in 500 µl of ice-cold 10% glycerol (10ml glycerol: 90ml distilled water). These competent cells were then stored in aliquots at -70°C.

2.10.2.2. Electroporation

Electroporator (Bio-Rad, USA) was used and electrocompetent cells were transformed with a plasmid (7µl) according to the protocol mentioned in the instrument manual. The electro-competent cells were thawed on ice. Then 7µl plasmid was added to 50µl of these cells and the sample was transferred to a cuvette. The conditions for electroporation were set according to the manufacturer's manual and the PULSE button was pressed. 250µl LB media (Annexure 2.2) was added to the cuvette after the electroporation. The sample was then transferred to a fresh Eppendorf and placed in an incubator at 37°C for 3 hours with constant shaking. This sample was used for streaking on LA plates containing suitable antibiotic(s) and 100µl from the same sample was used for inoculation in LB containing corresponding suitable selection antibiotic(s). The plates as well as the inoculated LB were kept overnight at 28°C.

2.10.3. *Agrobacterium tumefaciens* strain and pGWB5 containing *OmpK*

Agrobacterium tumefaciens strain GV3101 was transformed with a pGWB5 binary vector having our gene of interest. This transformed *Agrobacterium* was used for the transformation of *Solanum lycopersicum* L. var. Rio Grande. The T-DNA region of the pGWB5 binary vector consisted of our gene of interest i.e., *OmpK* with 35S

promoter and NOS terminator. The 3' end of the *OmpK* gene contains His-tags followed by GFP as shown in Figure 2.1.

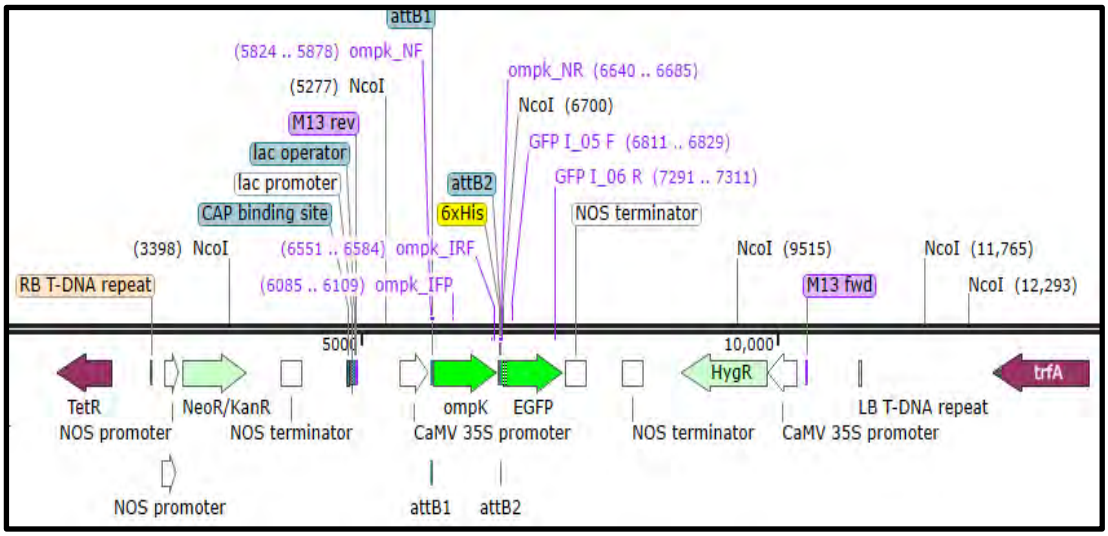

Figure 2.1: Schematic representation of final *OmpK* expression vector

2.11. Second transformation- Stable transformation of *Solanum lycopersicum* L.

Stable transformation of tomato (*Solanum lycopersicum* L.) cv. Rio Grande was performed using *Agrobacterium* transformed with pGWB5 containing the *OmpK* gene.

2.11.1. Preparation of bacterial culture

50ml of LB media (Annexure 2.2) having 50mg/l kanamycin and 50mg/l rifampicin for selection was taken and it was cultured with GV3101 strain of *Agrobacterium tumefaciens* containing the binary vector pGWB5 (having *OmpK*). The cultured flask was placed on an incubator shaker at 28°C for 48 hours. The OD_{600} was adjusted at 0.8 with liquid LB broth.

2.11.2. Co-cultivation

The inoculated LB media was shifted to a 50ml falcon and centrifuged at 4000 rpm at room temperature for 20 minutes. In the sterile environment of LFH, the supernatant was discarded, and the pellet was resuspended in 25ml of liquid ½ MS (Annexure 2.2). To this infection medium, 200 µM acetosyringone was added.

Explants (nodes) were cut from the tomato plant and dipped in this bacterial suspension for different infection times i.e., 8 and 10 minutes within the sterile conditions of LFH. After infection, they were put on autoclaved filter papers and

were shifted to the co-cultivation media plates (Annexure 2.3). 10-20 nodal explants were co-cultivated on each plate. The plates were sealed with parafilm and were kept in a growth room at 25±2°C in the dark for different time periods i.e., 2 and 3 days. Control (no infection) was also shifted to co-cultivation media (Annexure 2.3). The entire work was done in duplicate.

2.11.3. Selection

After 2 or 3 days of cocultivation, the explants were washed with washing media composed of freshly prepared liquid ½ MS (Annexure 2.2) and cefotaxime (500mg/l). Three washing steps were done using the same washing media and the explants were washed for 5 minutes in each step as described in Figure 2.2. After that, the final washing was done using autoclaved distilled water and the explants were air-dried using sterile filter paper. After drying, they were transferred to the selection plates (Annexure 2.3). These plates were sealed with parafilm and were placed in light in the growth room at 25±2°C. After two weeks, these infected explants were washed thrice by dipping in washing media for 5 minutes and shifted onto petri plates having fresh selection media.

The whole work was done in LFH under aseptic conditions. Transformed nodal explants of tomato were regenerated, and their Regeneration efficiency and callus formation efficiencies of explants were calculated by following formulas.

Regeneration efficiency (%) = (Number of shoots regenerated) / (Total number of explants inoculated) ×100

Callus formation efficiency (%) = (Number of calli produced from explants) / (Total number of explants inoculated) × 100

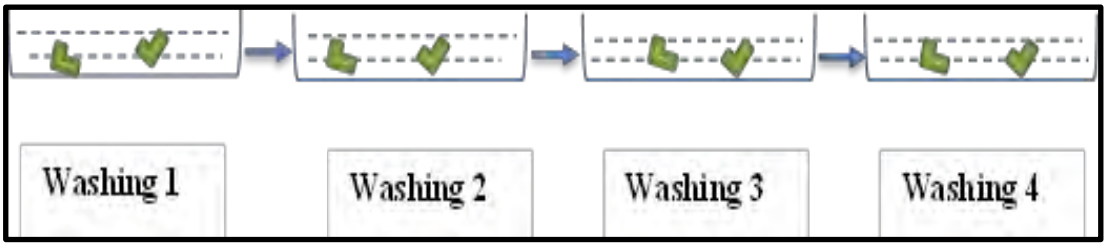

Figure 2.2: Washing steps after co-cultivation. Washing 1: Liquid ½ MS with 500mg/l cefotaxime; Washing 2: Liquid ½ MS with 500mg/l cefotaxime; Washing 3: Liquid ½ MS with 500mg/l cefotaxime; Washing 4: Autoclaved distilled water

2.12. Molecular biology techniques to verify transformation

Transformation of the tomato plant (*Solanum lycopersicum* L.) plant genome with the *OmpK* gene was confirmed by using different molecular biology techniques. Integration of the *OmpK* gene in the tomato plant genome was confirmed through conventional PCR. qRT-PCR was performed to check the exact copy number of the integrated gene.

For OmpK protein expression confirmation in transformed plants, western blot and Dot blot techniques were performed in which total soluble protein extracted from transformed tomato plants was used. To detect and quantify OmpK protein in transformed plants, indirect enzyme-linked immunosorbent assay (ELISA) was performed.

2.12.1. Plasmid isolation

Plasmid isolation was done by following the protocol reported by Sambrook and Rusell (2001), with few modifications.

The inoculated LB media (with bacterial growth of 0.6-0.8 $O.D._{600}$) was shifted to a fresh 50ml falcon. The culture was centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant was removed, and the pellet was dissolved in 200µl of solution I (Annexure 2.1) which was then transferred to a fresh Eppendorf tube. 400µl of solution II (Annexure 2.1) was added. The tube was gently inverted several times and was stored on ice for 3-5 minutes. 300µl of lysis solution III (Annexure 2.1) was added to it. All the components were mixed by inverting the tube several times. The tube was again incubated on ice for 5 minutes followed by centrifugation at 14000rpm for 5 minutes at 4°C. 600µl of the supernatant was collected and transferred to a fresh tube. An equivalent amount of phenol-chloroform (Annexure 2.1) was added to it. Again, the tube was centrifuged at maximum speed for 5 minutes at 4°C. Two layers were observed. The top aqueous layer was transferred to a fresh Eppendorf tube. To this, 600µl of isopropanol was added and the tube was stored on ice for 1 hour (60 minutes). After one hour, it was centrifuged at maximum speed for 5 minutes at 4°C. The supernatant was discarded. The pellet was washed using 70% ethanol (1ml) followed by centrifugation at the maximum speed for 5 minutes at 4°C. Ethanol was removed and the pellet was allowed to dry. Depending on the size of the pellet, it was dissolved in T.E buffer (Annexure 2.1) with RNase A and was stored at -20°C.

2.12.2. Extraction of plant genomic DNA

Genomic DNA was extracted by cetyltrimethylammonium bromide (CTAB) procedure (reported by Murray and Thompson, 1980). DNA was isolated from both wild-type and transgenic plants.

CTAB buffer (Annexure 2.4) was prepared one day before the DNA extraction from plants. The sample (nodes) was placed overnight at -20°C. The next day, it was ground into fine powder with liquid nitrogen. The powdered sample was transferred to freshly autoclaved Eppendorf. Water-bath temperature was pre-adjusted at 65°C. CTAB was placed in the water bath for 30-60 minutes before using it. 700µl of CTAB buffer was added to the sample in Eppendorf. The homogenate was vortexed and placed in the water bath (temperature 65°C) for an hour. After an hour, 600µl chilled phenol-chloroform-isoamyl alcohol (Annexure 2.4) was added to each sample. The components were mixed well by inverting the Eppendorf several times from time to time for 30 minutes. Then it was centrifuged for 15 minutes at 14,000 rpm. The supernatant was collected in a fresh Eppendorf and the pellet was discarded. An equal volume of chilled isopropanol was added to it and the sample was incubated overnight at -20°C. The very next day, the sample was centrifuged for 5 minutes at 14,000 rpm and the pellet was collected. 1ml chilled 70% ethanol (Annexure 2.4) was added to the pellet followed by centrifugation at 14,000 rpm for 2 minutes. The supernatant was discarded, and the washing step was repeated. The supernatant was discarded again. The pellet was allowed to air-dry by inverting it on a paper towel for 10-20 minutes. Finally, the pellet was dissolved in an appropriate amount of T.E buffer (Annexure 2.4) and RNase A (depending on the size of the pellet) and stored at -20°C.

2.12.3. Micro-volume quantification of DNA

NanoDrop micro-volume quantification, a highly sensitive technique, was used to assess the concentration of DNA isolated from the transformed tomato plant using a Colibri microvolume spectrophotometer. 1-2µl of de-ionized water was applied to the bottom optical panel of the microvolume spectrophotometer to clean it before analyzing the DNA sample. When the lever arm was completely closed, the upper panel came into touch with deionized water. The lever arm was then raised, and the two optical panels were cleaned using dry, clean, fur-free lab wipes. After that, the

NanoDrop software was opened, and the "Nucleic Acid application" option was chosen.

A blank measurement was performed by dispersing 1µl of TE buffer over the lower optical panel. Lowering the lever arm, "Blank" was selected as the option. The two optical panels were cleaned using a lab wipe once the blank measurement was finished. The appropriate constant was chosen to enable the quantification of DNA samples. 1µl of the DNA sample was placed over the lowest optical panel. After shutting the lever arm, the "measure" was chosen in the software program. The software automatically gave the ratio of DNA concentration and purity in a specified unit along with a graph.

2.12.4. Polymerase Chain Reaction (PCR)

For a successful transformation, integration of the *OmpK* gene in the transgenic tomato plant genome was checked and confirmed by conventional PCR reaction. Positive (*OmpK*-containing plasmid) and negative controls were used. Wild-type plants were used as a negative control. After extraction of DNA from transgenic and wild-type plants, they were employed in a PCR reaction with specific primers that would only amplify a certain region, giving us amplicons of 890 bp with *OmpK* nuclear primers and 500 bp length with *OmpK* internal primers.

***OmpK*-nuclear forward primer**

5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCGTAAATCACTTTTAGCTCTAGGCC**3'**

***OmpK*-nuclear reverse primer**

5'GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGATGGTGATGGTGATG**3'**

***OmpK* internal forward primer**

5'CTAAGCAACCCAAGCAGTGACAAAG**3'**

***OmpK* internal reverse primer**

5'CCTTCATCTTTCAGACCATAAACATCTTTGTAGC**3'**

The total PCR reaction had 35 repeated amplification cycles. The total volume of the reaction mixture was 25 µl. The composition of the master mix is given in the

following Table 2.10. PCR process with temperature and time at different stages of reaction is given in Figure 2.3. The annealing temperature was 66°C for *OmpK* nuclear primers and 56°C for *OmpK* internal primers.

Table 2.10: Composition of 1X reaction mixture for PCR

S.No.	Components	Concentration for 1X (µl)
1.	Taq buffer	2.5µl
2.	$MgCl_2$ (25mM)	1.5µl
3.	dNTPs	0.5µl
4.	Forward primer	0.5µl
5.	Reverse primer	0.5µl
6.	Taq polymerase	0.5µl
7.	Template (DNA)	1µl
8.	PCR water	18µl

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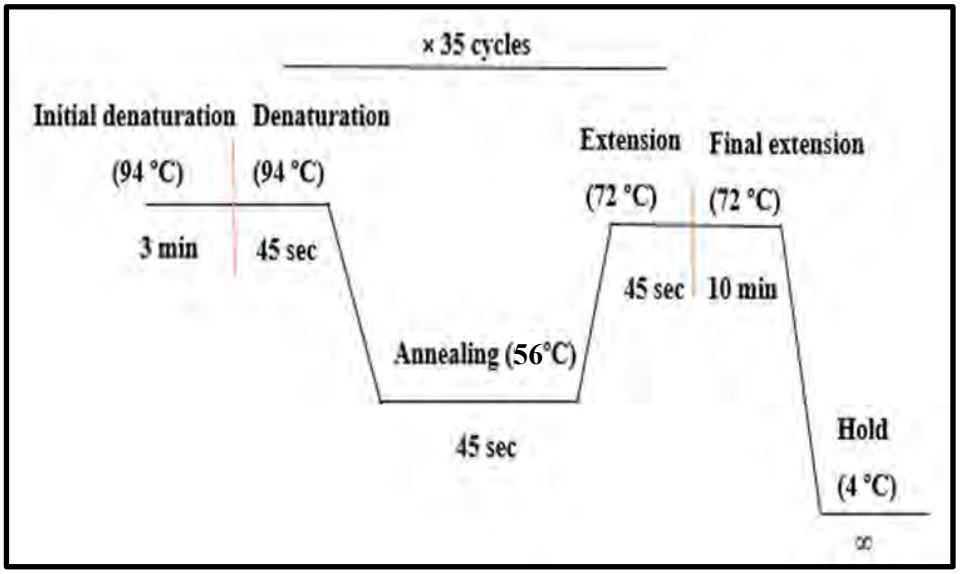

Figure 2.3: Profile of PCR program conditions required in different steps of a PCR reaction

2.12.5. Agarose Gel Electrophoresis

Agarose gel electrophoresis was done to check the presence of different fragments of DNA samples. Agarose creates pores that divide the various DNA fragments according to their sizes, allowing the smaller fragments to travel farther from the wells due to less resistance than the bigger ones. The concentration of agarose required to make the gel is dependent on the length of the DNA fragments that need to be separated, and this concentration of agarose determines the pore size. The concentration of agarose used to make the gel will increase as the size of the DNA fragment decreases and vice versa. As a result, the two factors are inversely related.

2.12.5.1. Preparation of Agarose gel

1% agarose gel was prepared in this research work in which 1g of agarose powder was added to 100ml of 1X TBE buffer (Annexure 2.5) in a reaction bottle. The solution was then heated in an oven to dissolve the agarose completely. Then 7µl of ethidium bromide (Annexure 2.5) was added. The gel was then poured into an assembled gel casting tray and a comb was inserted to create wells for loading the sample. It was allowed to set at room temperature. After the gel has solidified completely, the comb was removed, and the gel was placed in the gel tank containing 1X TBE buffer (Annexure 2.5).

2.12.5.2. Sample preparation

Before loading the samples on the gel, they were prepared by mixing them with loading dye (Annexure 2.5). The loading dye is composed of bromophenol blue and sucrose. As the DNA samples move towards the positive electrode on the gel, bromophenol blue helps to keep track of them, but sucrose binds to DNA, increases its density, enables it to settle more rapidly, and inhibits diffusion.

2.12.5.3. Electrophoresis and imaging

Agarose gel electrophoresis was used to confirm the PCR results. The wells were filled with all the PCR samples, including the transgenic and wild-type samples. Before loading the gel, 7µl of PCR products were mixed with 3µl of loading dye (Annexure 2.5). 1kb ladder (Cat No: SM0314, Thermo Scientific, USA) (Figure 2.4) was used for checking the size of the DNA sample. Each sample was loaded carefully into each well. Following the loading of the samples, the gel tank's lid had been closed, the power source was attached, and electrophoresis was carried out at

110 volts for 60 minutes. DNA bands were visualized under UV light after a successful run using the gel documentation system.

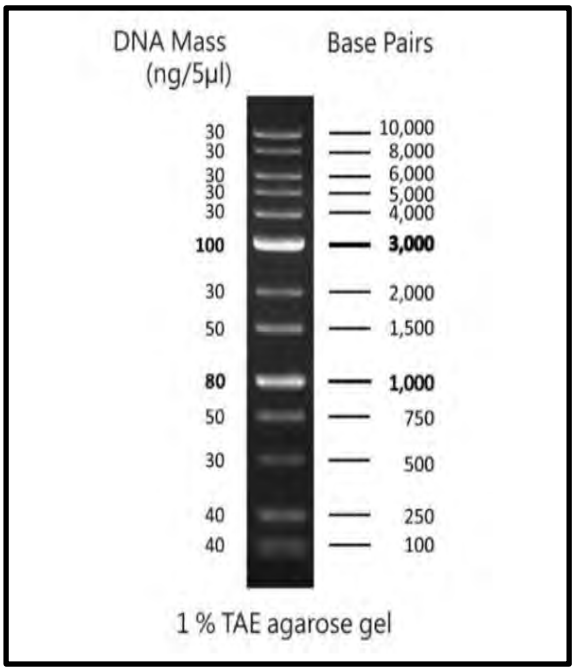

Figure 2.4: 1kb DNA ladder for agarose gel electrophoresis (Taken from: https://www.genedirex.com)

2.12.6. Quantitative Real Time (qRT) PCR

After successful transformation, we performed qRT-PCR for checking the level of expression of a transgene within a plant cell. The experiment was performed by following the protocol described by Wen *et al*. (2012).

qRT PCR was done using MyGo Pro Real-time PCR (Stokesley Middlesbrough, UK). We used DNA samples of both wild-type and transgenic. Three dilutions i.e., 1:10, 1:100, and 1:1000 was prepared in PCR water for each DNA sample. A fluorescent dye called SYBR Green (Cat No: K0221 Thermo Scientific, USA) was used in this technique. Master-mix was prepared as given in Table 2.11. The total reaction mixture was 10µl, unlike conventional PCR. 9.5µl of this reaction mixture was added to each sterile PCR tube and then 0.5µl of DNA sample was added. All the PCR tubes were properly labeled and placed in the instrument. The conditions for qRT-PCR are given in Table 2.12. The primers used are provided as follows.

***OmpK* internal forward primer**

5'CTAAGCAACCCAAGCAGTGACAAAG**3'**

***OmpK* internal reverse primer**

5'CCTTCATCTTTCAGACCATAAACATCTTTGTAGC**3'**

Table 2.11: Composition of the reaction mixture for qRT-PCR

S.No.	Components	Concentration for 1X (µl)
1.	Forward primer	0.25
2.	Reverse primer	0.25
3.	DNA	0.5
4.	SYBR Green	5
5.	PCR water	4

Table 2.12: Conditions for qRT-PCR

Programs name		Temperature (°C)	Ramp (°C/s)	Hold (s)
Hold		95	4	600
3 step amplifications	**Denaturation**	95	5	10
	Annealing	56	4	45
	Extension	72	5	15
Pre-melt hold		95	5	15
High-resolution melting	**Initial stage**	56	4	60
	Final stage	95	0.05	15

2.12.7. Protein extraction

For the extraction of protein, we used transformed plant tissue which was sliced into small pieces of 100mg weight. The pieces were ground into fine powder by using

liquid nitrogen, mortar, and pestle. We added 400µl of extraction buffer (Annexure 2.6) to this fine powder. A mixture was made and transferred into a freshly autoclaved Eppendorf tube. We centrifuged it at 20,000g for 10 minutes at 4°C. The supernatant was collected and put in a fresh Eppendorf tube. This collected supernatant was considered as the Total Soluble Protein (TSP) fraction which was centrifuged once more for 10 minutes at 20,000g and 4°C to remove cellular debris. The supernatant was collected and shifted to a fresh Eppendorf tube and was stored at -20°C till further use.

2.12.8. Bradford assay for protein quantification

After extraction of the protein from the plant, the concentration of TSP was checked by using Bradford's assay. Bovine Serum Albumin (BSA) was used as a standard to plot the standard curve. 2mg/ml stock solution of BSA was prepared and six different dilutions of BSA were prepared from the stock (Annexure 2.8). The protein extraction buffer (Annexure 2.6) was used as a blank.

We loaded 20µl of blank, BSA dilutions, and samples into separate wells of a 96-well plate. Then, 200µl of Bradford's reagent was added to each well-containing sample. The plate was then covered with aluminium foil and placed in the dark for 30-60 minutes. After incubation, absorbance was taken at 595nm using a spectrophotometer. The absorbance values of BSA dilutions were then used to plot a standard curve as shown in Figure 2.5. The concentrations of each protein sample were calculated from this standard curve using the TREND formula on an Excel sheet.

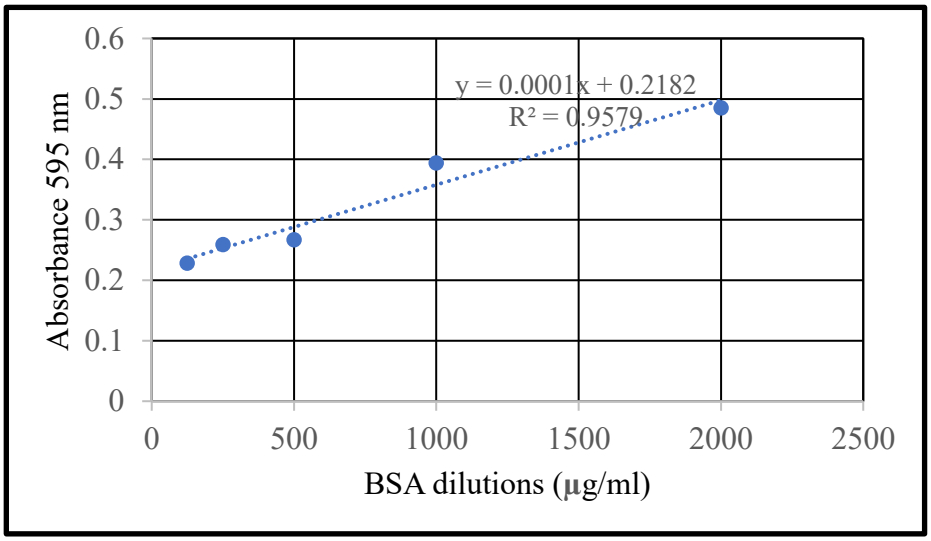

Figure 2.5: BSA standard curve for the quantification of protein.

2.12.9. Dot Blot assay

Dot Blot assay is performed for the identification and detection of proteins. In this technique, protein samples are detected as circular templates produced on the membrane directly. We used an antigen-specific primary antibody and an enzyme-conjugated secondary antibody for protein detection.

We performed Dot Blot for the detection of OmpK protein in the transgenic tomato plant.

2.12.9.1. Blotting

We used nitrocellulose membrane in the Dot Blot. A gridline was marked on the membrane using a pencil. At the mid of this line, 20µl samples of total soluble protein (TSP) were loaded like dots on the blot along with the wild type and then the membrane was allowed to dry completely.

2.12.9.2. Blocking

After the membrane had dried, blocking was done to block non-specific sites and non-specific binding of primary antibodies. A blocking solution (Annexure 2.6) was used. The membrane was kept dipped in the blocking solution for an hour at room temperature with continuous shaking. After 60 minutes, the blocking solution was removed, and the membrane was washed with wash buffer (Annexure 2.6) three times.

2.12.9.3. Primary antibody treatment

We used 6 X-His-tag antibodies (Invitrogen) as primary antibody in 1:10,000 ratio (Annexure 2.7). Working dilution of the primary antibody was prepared in TBS-T (Annexure 2.6) from stock. The membrane was dipped in primary antibody overnight at 4°C**.** After the treatment, it was washed three times using the wash buffer (Annexure 2.6).

2.12.9.4. Secondary antibody treatment

We used House-radish peroxidase (HRP) conjugated goat anti-mouse IgG as the secondary antibody. The membrane was placed in the secondary antibody (Annexure 2.7) for 1-2 hours at room temperature with constant shaking. Then, it was washed with the wash buffer (Annexure 2.6).

2.12.9.5. Visualization

For visualization, a Chemiluminescent substrate (Cat No: WBKLS0500, Merck Milipore, Germany) was used. Equal volumes of Luminol and hydrogen peroxide were mixed. The membrane was treated with the substrate for 2 minutes in the dark. After incubation, the blot was visualized using the gel documentation system (Alpha View SA Version 3.4.0.0).

2.12.10. Western Blotting

Firstly, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was performed for the separation of protein samples based on their sizes. And then they were transferred into the nitrocellulose membrane by using transferring assembly. After transferring, non-specific sites were blocked using the blocking buffer, and the membrane was treated with primary and secondary antibodies to detect the desired protein product.

We used protein samples from transgenic tomato plant tissue. Wild-type plants were used as the negative control.

2.12.10.1. SDS-PAGE

SDS PAGE was performed to separate protein samples based on their sizes. The apparatus for the electrophoresis was properly cleaned and assembled. In this, we prepared two gels i.e., 10% resolving gel and 5% stacking gel. Firstly, 10% Resolving gel (Annexure 2.6) was prepared and poured between the two assembled glass plates. To linearize the gel surface, 1 ml isopropanol was poured above the resolving gel and the gel was allowed to solidify and set completely. Then 5% stacking gel (Annexure 2.6) was prepared. Isopropanol was removed completely followed by the pouring of stacking gel above the resolving gel. The comb was inserted immediately after pouring, and the gel was allowed to set.

2.12.10.2. Protein sample preparation and loading

For the preparation of protein samples, we used the 4X sample buffer (Annexure 2.6). 25µl samples were mixed with 6µl sample buffer in a sterile Eppendorf tube to make 30 µl. Each sample along with the wild type was prepared in the same way and was heated in a water bath (95°C maintained temperature) for 10 minutes at 95 °C to denature the proteins. After the gel got set, the gel assembly was placed in the gel

tank. The tank was filled with a running buffer (Annexure 2.6). The comb was removed followed by washing of wells using running buffer before the samples were loaded in the wells. The samples were loaded into separate wells. In one well, a 3µl pre-stained PAGE ruler (Cat No. 26616, Thermo Scientific, USA) was loaded.

2.12.10.3. Electrophoresis

After the samples were loaded, the lid of the gel tank was closed, and the positive and negative electrodes of the gel tank were connected to the power supply. Initially, the gel was run for 20 minutes at 80 volts and then the voltage was increased to 110 volts for 90 minutes or till the gel was fully run. After the gel has run, western blotting was performed.

2.12.10.4. Transfer of protein

For western blotting, we transferred protein samples ran on the gel into the nitrocellulose membrane. For the transfer, we used the Semi-dry method using a semi-dry blotting apparatus. For one gel transfer, twelve Whatman filter papers were cut into equal sizes of the separating gel. These filter papers and nitrocellulose membrane was soaked in 1X transfer buffer (Annexure 2.6). The Sandwich was prepared by sequentially placing six filter papers, nitrocellulose membrane, separating gel, and the remaining six filter papers from the positive terminal (anode) to the negative terminal (cathode) as shown in Figure 2.6. A roller was used to remove air bubbles and the terminals of the transfer apparatus were connected to a power supply. The voltage was set to 10 volts for 45 minutes.

Figure 2.6: Transfer assembly for transfer of one gel

2.12.10.5. Blocking

After the transfer, blocking was done to block non-specific sites and non-specific binding of primary antibody. The blocking solution (Annexure 2.6) was used. The

membrane was kept dipped in the blocking solution for an hour at room temperature with continuous shaking. After 60 minutes, the blocking solution was removed, and the membrane was washed with wash buffer (Annexure 2.6) three times with continuous shaking.

2.12.10.6. Treatment with primary antibody

We used 6 X-His-tag antibodies (Invitrogen) as primary antibody in 1:10000 ratio (Annexure 2.7). Working dilution of the primary antibody was prepared in TBS-T (Annexure 2.6) from stock. The membrane was dipped in primary antibody overnight at 4°C**.** After the treatment, it was washed three times using a wash buffer (Annexure 2.6).

2.12.10.7. Treatment with secondary antibody

We used House-radish peroxidase (HRP) conjugated goat anti-mouse IgG as the secondary antibody. The membrane was placed in the secondary antibody (Annexure 2.7) for 1-2 hours at room temperature with constant shaking. Then, it was washed with the wash buffer (Annexure 2.6).

2.12.10.8. Visualization

For visualization, a Chemiluminescent substrate (Cat No: WBKLS0500, Merck Milipore, Germany) was used. Equal volumes of Luminol and hydrogen peroxide were mixed. The membrane was treated with the substrate for 2 minutes in the dark. After incubation, the blot was visualized using the gel documentation system (Alpha View SA Version 3.4.0.0).

2.12.11. ELISA

To check the concentration of proteins within the samples, indirect ELISA was done. A 96-well plate was used for doing indirect ELISA.

ELISA extraction buffer was used to prepare samples for ELISA (Annexure 2.9). The prepared protein samples were loaded into separate wells and the ELISA plate was incubated for overnight at 4°C. The ELISA plate was washed three times with wash buffer after an hour (Annexure 2.9). After that, to each well, 200µl of blocking solution (Annexure 2.6) was added. Again, incubation of 1 hour at 37°C was given to the plate. After an hour, the blocking solution was removed and then the wells were washed three times with the wash buffer (Annexure 2.9).

After washing, 50µl primary antibodies (Annexure 2.7) were added to each well and the plate was incubated at 37°C for an hour. The wells were washed thrice with wash buffer (Annexure 2.9) and then in each well, the secondary antibody (Annexure 2.7) was added. The plate was placed in an incubator at 37°C for 1 hour.

Again, wash the wells thrice with wash buffer (Annexure 2.9) so that the unbound antibody was removed. Enzyme-specific substrate, called TMB (Cat No. A3840, Applichem, Germany), was added to each well and the plate was placed at room temperature. As the blue color was developed after 20-30 minutes, 0.1 N H_2SO_4 (stop solution) was added and then the absorbance was measured at 595 nm.

ANNEXURES

Annexure 2.1

Composition of Plasmid isolation solutions

Solutions	Components	Concentration
Solution I	Glucose	50mM
	Tris	25mM, pH=8
	EDTA	10mM, pH=8
Solution II	SDS	1%
	NaOH	0.2N
Solution III	Sodium acetate	3M, pH=4.8
	Glacial acetic acid	10%
Phenol-chloroform	Phenol	25ml
	Chloroform	25ml
70% Ethanol	Ethanol	70ml
	Distilled water	30ml
T.E buffer	Tris HCL (pH=8)	1M
	EDTA	0.5M

Annexure 2.2

Reagents for culture media

S.no	Media	Components	Concentration (g/l)
1.	½ MS	MS basal salts	2.2g
		Sucrose	30g
		Agar	8g (0.8%)

2.	Full MS	MS vitamins	4.4g
		Sucrose	30g
		Agar	8g (0.8%)
3.	Liquid ½ MS	MS basal salts	2.2g
		Sucrose	30g
4.	LA	LB broth	25g
		Bacterial Agar	1g (0.1%)
5.	LB	LB broth	25g

Annexure 2.3

Media required for transformation

Media	Components	Concentration
Co-cultivation media	MS Vitamins	4.4g/l
	Sucrose	30g/l
	Agar	0.8%
	Zeatin	2mg/l (1000µl/L)
	Acetosyringone	200µM (2000µl/L)
Washing media	Liquid ½ MS	2.2 g/l MS, sucrose 30g/l
	Cefotaxime	250mg/l
Selection media	MS Vitamins	4.4g/l
	Sucrose	30g/l
	Agar	0.8%
	Zeatin	2mg/l (1000µl/L)
	Hygromycin	25mg/l (500µl/L)

	Cefotaxime	250mg/l (1000µl/L)

Annexure 2.4

Solutions for DNA extraction from plants

Chemicals	Composition	Concentrations
CTAB buffer	CTAB	2%
	Tris-HCl	100mM
	EDTA	20mM
	NaCl	1400mM
	PVP	1%
Phenol-chloroform-isoamyl	Phenol	25ml
	Chloroform	24ml
	Isoamyl alcohol	1ml
70% Ethanol	Ethanol	70ml
	Water	30ml
T.E buffer	Tris HCL (pH=8)	1M
	EDTA	0.5M

Annexure 2.5

Solutions for Gel Electrophoresis

Solutions	Composition	Concentrations
10X TBE buffer recipe pH=8.0	Tris base	110g/l
	Boric acid	55g/l
	EDTA	9.3g/l
1X TBE buffer	10X TBE buffer	10ml

	Distilled water	90ml
Ethidium Bromide 10mg/ml	Ethidium Bromide	1g
	Distilled water	100ml
Loading dye	Bromophenol blue	0.25g
	Distilled water	100ml

Annexure 2.6

Solutions for Western Blotting

Solutions	Composition	Concentrations
Protein extraction buffer	Sodium chloride	30mM
	Ascorbic acid	20mM
	Sodium metabisulphite	10mM
	Tris (pH=08)	100mM
Resolving gel	Distilled water	17.6ml
	30% stock acrylamide solution	35.25ml
	4X Resolving Tris solution	17.5ml
	10% Ammonium per sulphate	700µl
	TEMED	70µl
Stacking gel	Distilled water	16.8ml
	30% stock acrylamide solution	2.6ml
	4X Stacking Tris solution	6.4ml
	10% Ammonium per sulphate	178µl
	TEMED	26.8µl
4X Sample buffer	Glycerol	4ml
	B-mercaptoethanol	2ml

	SDS	1.2g
	4X stacking Tris	5ml
	Bromophenol blue	0.03g
Running buffer	Tris HCl	3g
	Glycine	14.4g
	SDS	1g
	Distilled water	1L
Semi-Transfer buffer	Tris-base	3.028g
	Glycine	14.4g
	Methanol	200ml
	Distilled water	Up to 1L
Blocking buffer	BSA	5g
	TBS-T	100ml
Wash buffer TBS-T	Tris-HCl	3.08g/l (pH=7.6)
	NaCl	8.775g/l
	Tween-20	1ml/l
	Distilled water	1L
Stock 30% Acrylamide gel solution	Acrylamide	30g
	Methylene bisacrylamide	0.8g
	Distilled water	100ml
Stock Resolving gel Tris (pH=8.8)	Tris HCl	36.4g
	SDS (10%)	8ml
	Distilled water	200ml
Stock Stacking gel	Tris-base	12.12g

Tris (pH=6.8)	SDS (10%)	8ml
	Distilled water	200ml
10% SDS	SDS	10g
	Distilled water	100ml

Annexure 2.7

Antibody dilutions

Solutions	Composition	Concentrations
His tag Primary antibody dilution (1:10000)	Primary antibody	3µl
	Sodium azide	0.015g
	BSA	1.5g
	TBS-T	30ml
HRP-conjugated goat anti-mouse IgG Secondary antibody dilution (1:10000)	Secondary antibody	3µl
	BSA	1.5g
	TBS-T	30ml

Annexure 2.8

BSA dilutions for Bradford assay

Concentration (µg/ml)	BSA (µl)	Distilled water (µl)
0	0	250
125	31.25	250
250	62.5	250
500	125	250
1000	250	250

2000	500	0

Annexure 2.9

ELISA solutions

Solutions	Composition	Concentrations
ELISA extraction buffer	$MgCl_2$	5mM
	NaCl	1M
	$CaCl_2$	5mM
	HEPES-free acid	20 mM (pH= 7.4)
	MSF	1mM
	Triton X-100	0.01%
Wash buffer (TBS-T)	Tris-HCl	3.08g (pH=7.6)
	NaCl	8.775g
	Tween-20	1ml
	Distilled water	1L

RESULTS

In this study, optimization of the hygromycin concentration for the selection of transformed plant using wild-type *Solanum lycopersicum* L. plant was done, best conditions for the transformation of nodal explants of *Solanum lycopersicum* L. were optimized, and an efficient protocol for stable *Agrobacterium*-mediated transformation of *Solanum lycopersicum* L. cv. Rio Grande. with *OmpK* gene was developed. Additionally, Polymerase Chain Reaction (PCR) conditions for the *OmpK* gene were optimized. Confirmation of integration and expression of the *OmpK* gene was done using different molecular biology techniques i.e., conventional PCR and quantitative real-time polymerase chain reaction (qRT-PCR). Dot blot, Western blot, and ELISA were done to validate the presence of OmpK protein.

3.1. Seed germination

In this study, all research work was carried out by using plants germinated from *Solanum lycopersicum* L. (Tomato) cv. Rio Grande seeds.

3.1.1. Sterilization and germination media for *Solanum lycopersicum* L. cv. Rio Grande seeds

Tomato seeds were surface sterilized using 0.1% $HgCl_2$ solution by just dipping the seeds in it for 15 to 20 seconds, followed by three times washing with distilled water. The seeds were inoculated on both strengths of MS media i.e., ½ MS and full MS media, and were observed for 30 days of inoculation as shown in Figure 3.1.

3.1.2. Germination efficiency of tomato seeds

Sterilized seeds were grown in ½ MS and full MS media within the sterile conditions of LFH. The experiment was done in triplicates. A total of 40 seeds were used. 20 were inoculated on ½ MS media and 20 on full MS. Each jar consisted of 6 or 7 seeds. The germination efficiency for seeds inoculated was calculated after 30 days of inoculation of seeds in both media.

From the results, it was observed that seeds sterilized with 0.1% $HgCl_2$ on ½ MS media had a high germination rate. The results showed that the germination efficiency was the same on both media i.e., 80% as shown in Table 3.1 and represented graphically in Figure 3.2 but the germination rate of seeds was quite different. It was observed that seeds inoculated on ½ MS media germinated faster compared to the

ones on full MS as shown in Figure 3.1. Thus, for later experiments, tomato seeds were inoculated in ½ MS media.

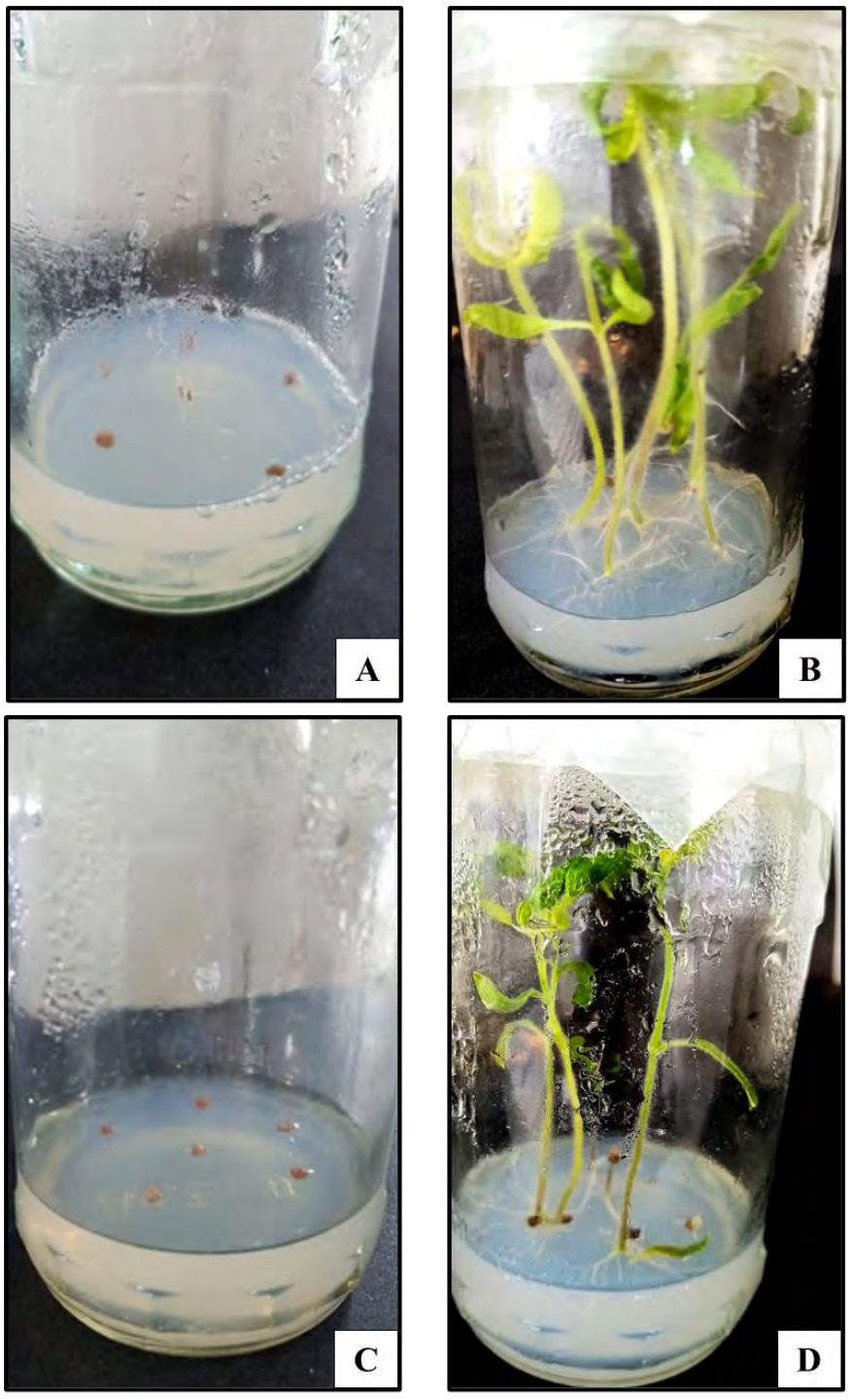

Figure 3.1: Tomato cv**.** Rio Grande seeds germinated on different strengths of MS media (½ MS and full MS media). (A). Day 2 on ½ MS (B). Day 30 on ½ MS (C). Day 2 on full MS (D). Day 30 on full MS

Table 3.1: Germination efficiency of *Solanum lycopersicum* L. on different MS strengths

S.No.	MS Strength	Total seeds inoculated	Seeds germinated	Germination efficiency
1.	½ MS	20	16	80%
2.	Full MS	20	16	80%

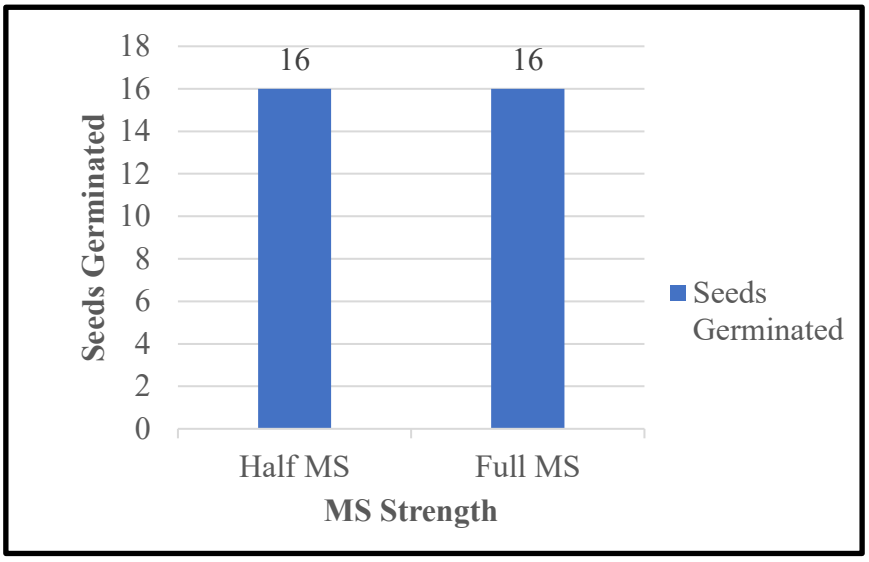

Figure 3.2: Graphical representation of seeds germination efficiency on both MS strengths (½ MS vs full MS media). The data is from three batches

3.2.Tomato plant regeneration

3.2.1. Regeneration efficiency of tomato explants

Tomato nodal explants were regenerated on optimized regeneration media (Full MS with vitamins along with a calculated amount of zeatin). The experiment was done in triplicates. After inoculation, the explants were observed for 4 weeks. Shoot regeneration was observed in most of the nodal explants as shown in Figure 3.3. Firstly, the callus was formed in almost each of the nodal explants after which shoot development was observed in most of them. Moreover, root development was also observed in the same regeneration media. Regeneration efficiency for *Solanum lycopersicum* L. nodal explants is calculated after 30 days of the inoculation of explants, which is tabulated in Table 3.2.

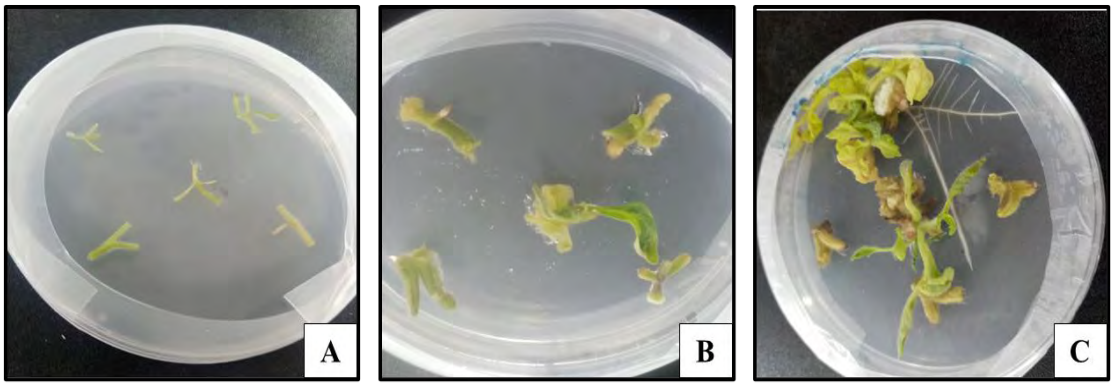

Figure 3.3: Regeneration of tomato nodal explants on optimized regeneration media (Full MS media with zeatin). Nodal explants were observed for 3 weeks (A). Day 2 on regeneration media (B). Day 15 on regeneration media (C). After Day 30 on regeneration media

Table 3.2: Regeneration efficiency of *Solanum lycopersicum* L. nodal explants

Type of explant	Total no. of nodes inoculated	No. of nodes regenerated	No. of callus formed	Regeneration efficiency	Callogenesis efficiency
Nodes	15	11	15	73.33%	100%

3.3. Effect of Hygromycin concentration on the survival of *Solanum lycopersicum* L. explants

To determine the optimal concentration of hygromycin for the selection of transgenic explants, wild-type explants of *Solanum lycopersicum* L. cv. Rio Grande was prepared and shifted to different concentrations of hygromycin to check its effect on these explants. The concentration of hygromycin that bleaches out wild-type explants was used later for the selection of transformed explants.

After preparation, explants were inoculated on optimized regeneration media stated above with different concentrations of hygromycin i.e., 0mg/l, 10mg/l, 15mg/l, 20mg/l, and 25mg/l, and observed for 4 weeks. It was noticed that with the increase in the concentration of hygromycin, the regeneration ability decreases, and more explants tend to bleach out. Figure 3.4 shows the effect of different concentrations of hygromycin on tomato explants with the results tabulated in Table 3.3. A graphical representation of these results is given in Figure 3.5.

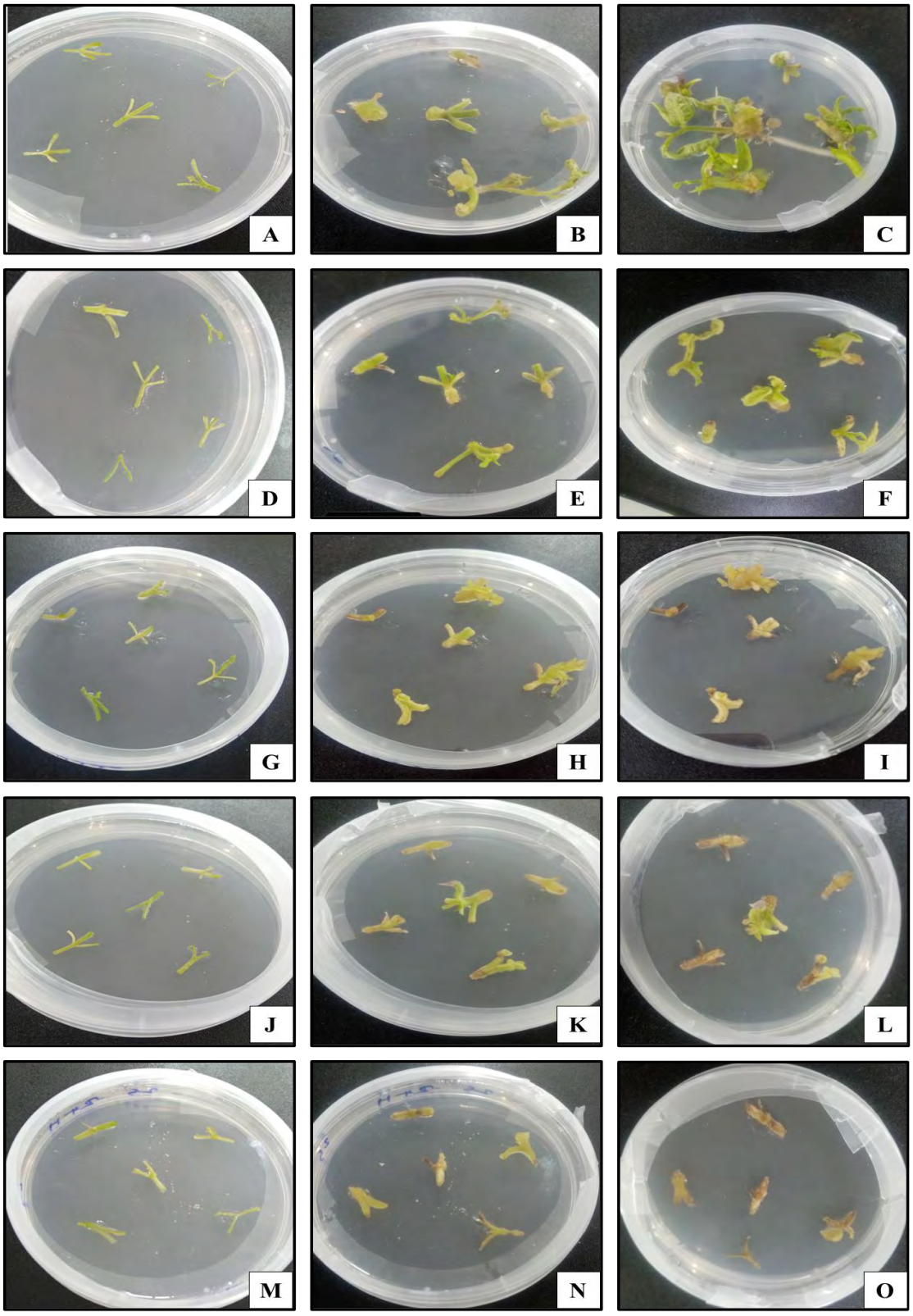

Figure 3.4: Effect of hygromycin concentrations on nodal explants of *Solanum lycopersicum* L. cv. Rio Grande. Different concentrations i.e., 0mg/l, 10mg/l, 15mg/l, 20mg/l, and 25mg/l of hygromycin were added to optimized regeneration media, and its effect was recorded on different days. (A). Day 2 on 0mg/l hygromycin (B). Day

15 on 0mg/l hygromycin (C). Day 30 on 0mg/l hygromycin (D). Day 2 on 10mg/l hygromycin (E). Day 15 on 10mg/l hygromycin (F). Day 30 on 10mg/l hygromycin (G). Day 2 on 15mg/l hygromycin (H). Day 15 on 15mg/l hygromycin (I). Day 30 on 15mg/l hygromycin (J). Day 2 on 20mg/l hygromycin (K). Day 15 on 20mg/l hygromycin (L). Day 30 on 20mg/l hygromycin. (M). Day 2 on 25mg/l hygromycin (N). Day 15 on 25mg/l hygromycin (O). Day 30 on 25mg/l hygromycin

Table 3.3: Germination efficiency of *Solanum lycopersicum* L. on different MS strengths

S. No.	Hygromycin Concentration (mg/l)	Total no. of explants	Explants regenerated	Explants bleached	Percentage of explants regenerated	Percentage of explants bleached
1.	0	15	11	0	73.33%	0%
2.	10	15	9	6	60%	40%
3.	15	15	6	9	40%	60%
4.	20	15	2	13	13.33%	86.67%
5.	25	15	0	15	0%	100%

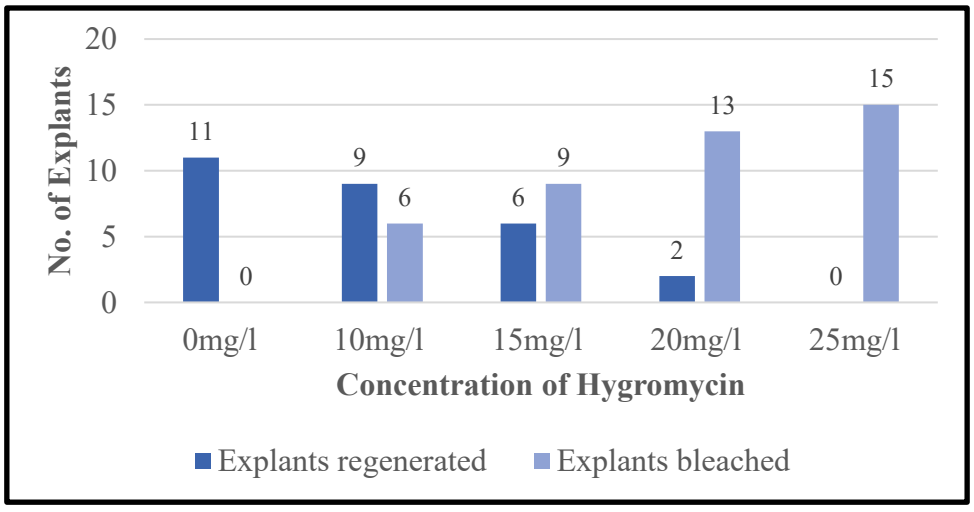

Figure 3.5: Graphical representation of hygromycin concentration effect on the regeneration of explants. The graph represents data from three batches (n=3)

3.4. Transformation

The OmpK gene present in the pGWB5 binary vector was transformed in the *Agrobacterium* strain GV3101. Plasmid isolation was performed, and conventional PCR was done to confirm this transformation. Then nodal explants of tomato were transformed with the *OmpK* gene (in pGWB5 binary vector) via *Agrobacterium*-mediated transformation. Integration of the *OmpK* gene into the plant genome was confirmed using conventional PCR. Gene expression and protein levels were confirmed using different molecular techniques.

3.4.1. PCR confirmation of plasmid containing *OmpK* gene

Plasmid isolation protocol by Sambrook and Rusell (2001) was used to isolate plasmid. The plasmid was isolated after the transformation of PGWB5 containing the *OmpK* gene transformed in the *Agrobacterium* strain GV3101. This isolated plasmid was confirmed by agarose gel electrophoresis. The presence of the *OmpK* gene in the plasmid was confirmed by conventional PCR by using specific primers for amplification of the transgene. The PCR product was loaded onto an agarose gel for confirmation of gene of interest. *OmpK* internal forward and reverse primers were used for the confirmation of the *OmpK* gene in the plasmid. Gel results showed the presence of the *OmpK* gene in the plasmid isolated (Figure 3.6).

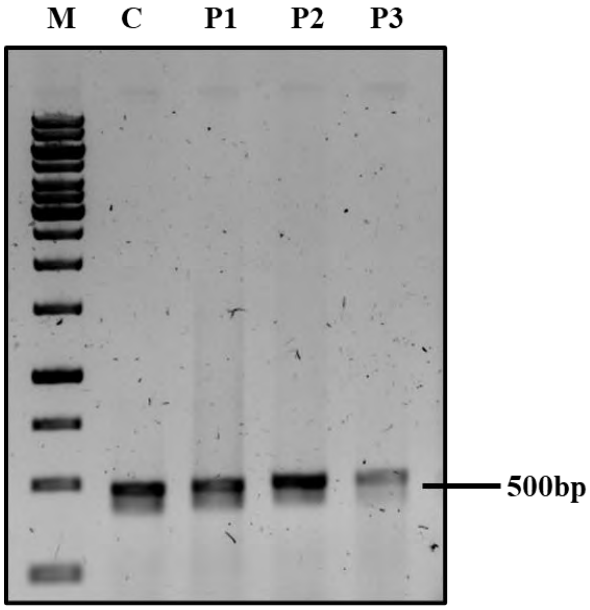

Figure 3.6: PCR confirmation of *OmpK* gene in plasmid. PCR confirmation of *OmpK* gene with *OmpK* internal forward and reverse primers (M). 1Kb Marker (C). Positive control (P1, P2, P3). Plasmids

3.4.2. *Agrobacterium*-mediated Stable transformation of *Solanum lycopersicum*

Nodes of *Solanum lycopersicum* L. cv. Rio Grande was used for transformation due to their greater ability to regenerate. Explants were prepared by cutting them into smaller pieces. *Agrobacterium tumefaciens* with pGWB5 having the *OmpK* gene was used to infect these nodal explants. After infection, nodes were shifted to co-cultivation plates before transferring them to hygromycin plates.

3.4.2.1. Optimization of infection time

In this experiment, nodes were divided into two groups. Both groups were infected with *Agrobacterium*. One group of nodes were treated with bacteria culture for 8-10 minutes while the other group treated for 15 minutes. The explants were observed for some days (Figure 3.7). It was noticed that with the increase in the infection time, it gets more difficult to get rid of excess bacteria and the regeneration or transformation efficiency of nodes also reduces. Thus, 8-10 minutes was considered as an optimum infection for the transformation of nodal explants. Results regarding the infection time are tabulated in Table 3.4 and graphically represented in Figure 3.8.

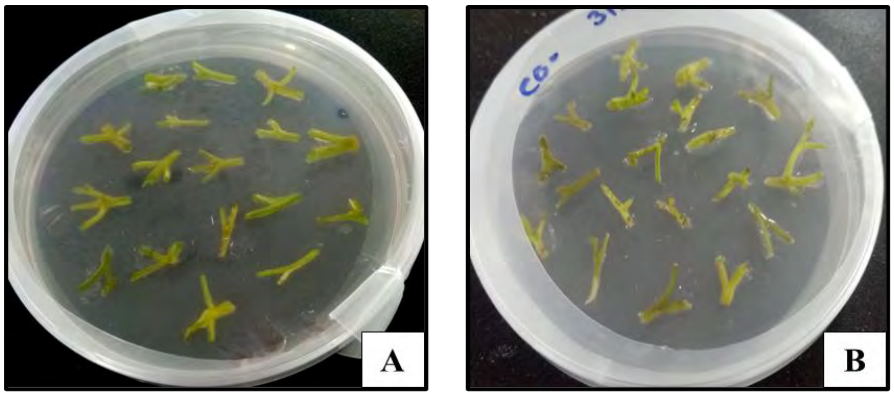

Figure 3.7: Nodal explants after infection on co-cultivation media. Explants were treated with *Agrobacterium tumefaciens* culture for different time periods. (A). Explants treated for 8-10 minutes (B). Explants were treated for 15 minutes

Table 3.4: Effect of infection time on regeneration/transformation of tomato explants

Infection time	Total nodes	Nodes regenerated	Transformation efficiency
8-10 minutes	25	15	60%
15 minutes	25	5	20%

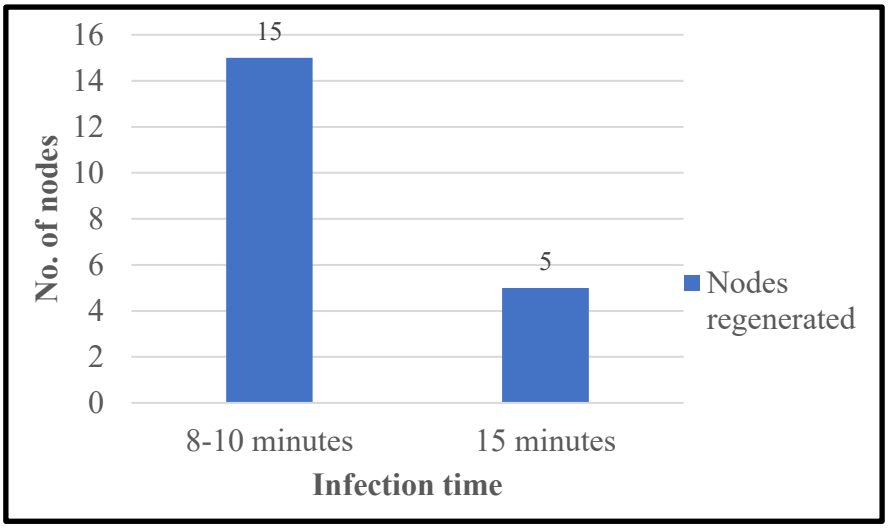

Figure 3.8: Graphical representation of the effect of infection time on regeneration/transformation efficiency of nodes (explants)

3.4.2.2. Optimization of co-cultivation time

After infection, explants were shifted onto co-cultivation media and were kept in the dark for 2 and 3 days. After the respective co-cultivation time, the explants were observed. It was noticed that with the increase in the co-cultivation time, decreased regeneration or transformation efficiency of nodes was observed. A higher regeneration potential was observed in explants inoculated for 2 days on co-cultivation media than the ones with co-cultivation time of 3 days as shown in Figure 3.9. The results for optimization of co-cultivation time are tabulated in Table 3.5 with a graphical representation provided in Figure 3.10.

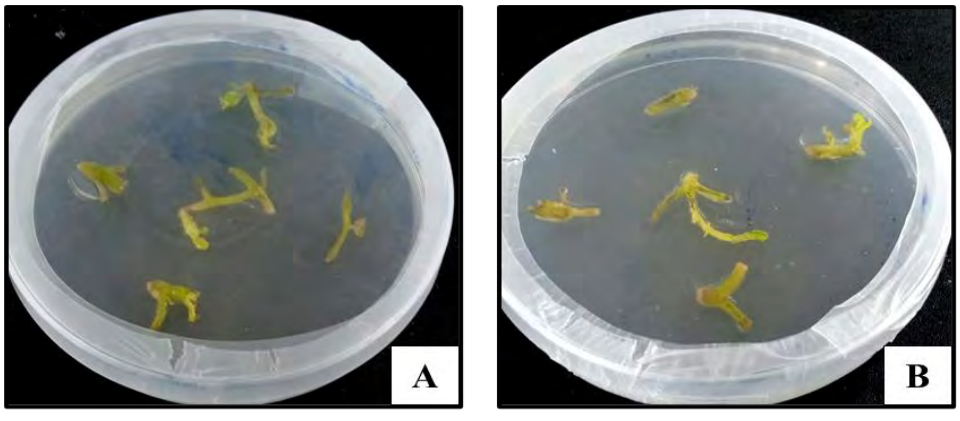

Figure 3.9: Nodal explants after co-cultivation time. Different co-cultivation time was provided for nodal explants. (A). Explants after 2 days of co-cultivation time (B). Explants after 3 days of co-cultivation time

Table 3.5: Effect of co-cultivation time on regeneration or transformation efficiency of tomato explants

Co-cultivation time	Total no. of nodes	Nodes regenerated	Regeneration/Transformation efficiency
2 days	25	15	60%
3 days	25	5	20%

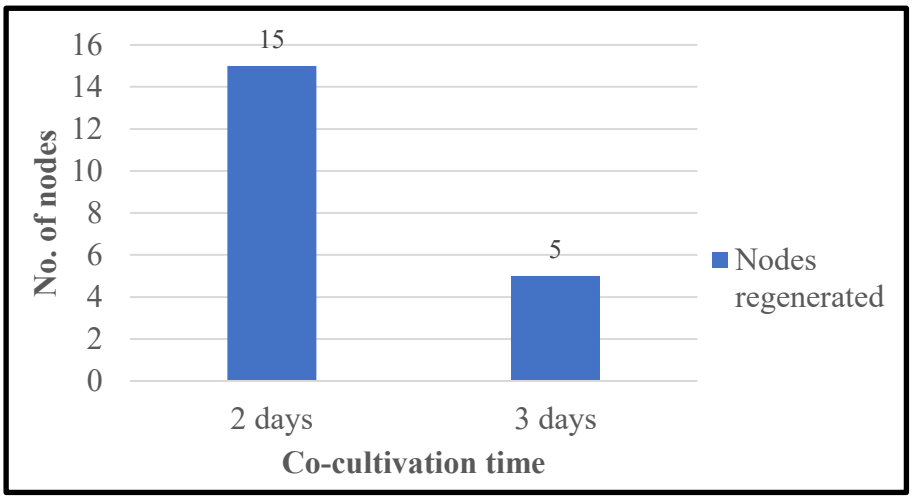

Figure 3.10: Graphical representation of the effect of co-cultivation time on regeneration or transformation efficiency of nodes (explants)

From the results obtained, it was concluded that with the infection time of only 10 minutes and co-cultivation time of 2 days maximum transformation efficiency in nodal explants was observed. Thus, these optimum conditions were used to transform nodal explants of *Solanum lycopersicum* L. cv. Rio Grande.

3.4.2.3. Stable Transformation

Following the optimization of several factors, *Agrobacterium*-mediated transformation was carried out under the optimized conditions. Nodes were infected with *Agrobacterium* containing the *OmpK* gene for 10 minutes. Treated samples were placed in the dark for 2 days (co-cultivation period). After 2 days, explants were washed with liquid ½ MS containing cefotaxime to remove excess bacteria. They were then shifted to selection plates containing hygromycin. This batch is considered batch 1 with optimized conditions and it is compared with batch 2 (with 3 days of co-

cultivation time). Figure 3.11 compares the transformation of batch 1 with the optimized conditions with batch 2 i.e., with 3 days of co-cultivation time. Transformation efficiency came out to be 60% in batch 1 and 20% in batch 2 as tabulated in Table 3.5 with a graphical representation provided in Figure 3.10.

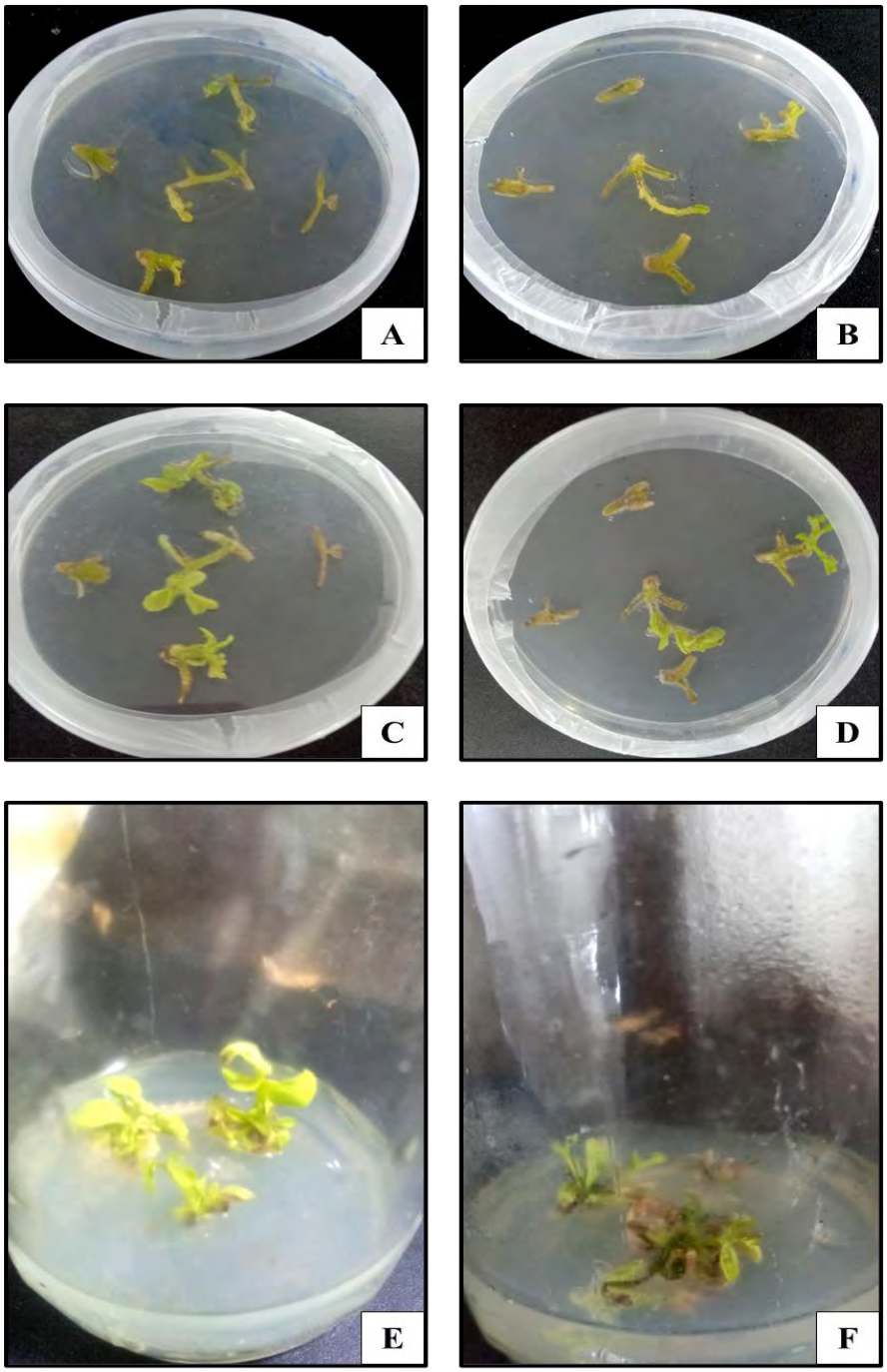

Figure 3.11: Nodal explants after co-cultivation time and washing on hygromycin selection plates. Two transformation batches were compared with different co-cultivation times i.e., transformation batch 1 with 2 days co-cultivation time and

transformation batch 2 with 3 days co-cultivation time (A). Nodal explants of transformation batch 1 after washing on hygromycin selection plates (B). Nodal explants of transformation batch 2 after washing on hygromycin selection plates (C). Transformation batch 1 after 15 days on selection plates (D). Transformation batch 2 after 15 days on selection plates (E). Transformation batch 1 after 21 days on selection plates (F). Transformation batch 2 after 21 days on selection plates

3.5. Confirmation of stable transformation of *Solanum lycopersicum* L. with *OmpK*

3.5.1. PCR confirmation of transgene

Plant genomic DNA was extracted by the CTAB method as explained by Murray and Thompson (1980). Before PCR confirmation, extracted DNA was checked through agarose gel electrophoresis (Figure 3.12). The presence of the *OmpK* gene in *Solanum lycopersicum* L. was confirmed by conventional PCR by using specific primers for amplification of the transgene. *OmpK*-nuclear forward and reverse primers as well as *OmpK* internal forward and reverse primers were used for the confirmation of *OmpK* transgene. The PCR product was loaded onto an agarose gel for confirmation of our gene of interest. Figure 3.13 shows the gel image with amplified bands of 890kb with *OmpK*-nuclear forward and reverse primers and 500 kb with *OmpK* internal forward and reverse primers, which confirms the presence of *OmpK* transgene. Resultantly, the successful transformation of *Solanum lycopersicum* L. cv. Rio Grande plant was confirmed.

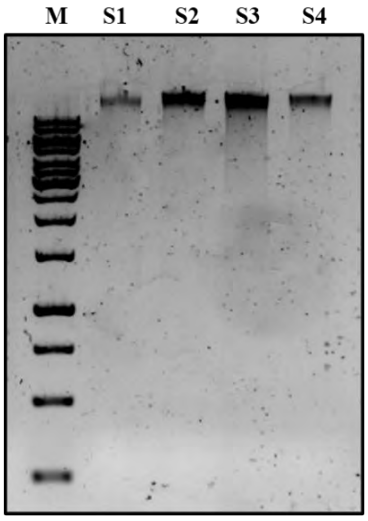

Figure 3.12: Confirmation of DNA isolated from plant samples. (M) 1 kb marker (S1). Sample 1 (S2). Sample 2 (S3). Sample 3 (S4). Sample 4

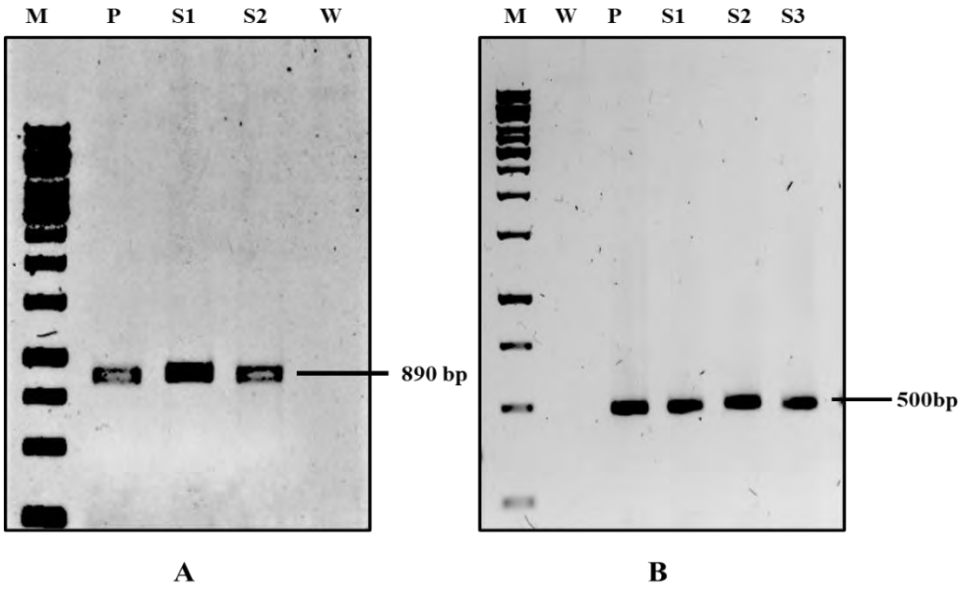

Figure 3.13: PCR confirmation of *OmpK* transgene in *Solanum lycopersicum* L. (A). PCR confirmation of *OmpK* transgene with *OmpK*-nuclear forward and reverse primers (B). PCR confirmation of *OmpK* transgene with *OmpK* internal forward and reverse primers (M). 1Kb Marker (W). Negative control wild-type plant (P). Positive control (S1, S2, S3). Transformed plant DNA samples

3.5.2. Calculation of *OmpK* copy number in transgenic *Solanum lycopersicum* L. by qRTPCR

Copy number of all transformed samples was calculated by following the protocol established by Schmittgen and Livak (2008). CQ values of endogenous gene and transgene were obtained (Table 3.6) which was used to plot the standard curve.

Table 3.6: Average CQ values of transgene vs endogenous gene

Sample	Average CQ values for *OmpK* transgene	Average CQ value for β actin
OmpK	32.97691693	29.82290853
OmpK 1:10	40.93300046	31.82694862
OmpK 1:100	39.712608	32.77485378
OmpK 1:1000	40.28996551	33.31442407

From these CQ values starting quantities (SQ) for transgene and endogenous genes were calculated which were 4.213563912 and 3.343672184, respectively. SQ values were used to calculate the rline which ultimately provided us with the copy number of the transgene by using the formula:

δr line= rline [(δsq trans/Sqtrans)^2+(δSQend/Sqend)^2)]^1/2

Copy number of *OmpK* transgene was equal to 2.

3.5.3. Dot Blot

Before western blotting, a Dot blot was done to check the presence of protein within the samples. Specific antibodies were used to detect the OmpK protein. Both wild-type and transformed samples were spotted on the blot. Unlike wild-type, positive results were observed for both samples (Figure 3.15). This confirmed the presence of transgenic protein in the samples.

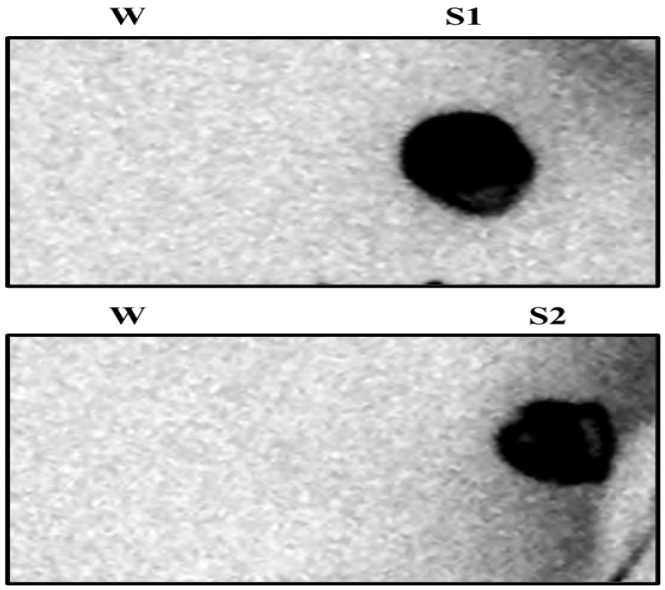

Figure 3.15: Confirmation of the presence of OmpK protein in transformed samples by Dot Blot. (W). Wild type (S1). Sample 1 (S2). Sample 2

3.5.4. Western blotting

The presence of OmpK protein was confirmed through western blotting. In the transgenic samples, 61 kDa (dimeric form) OmpK Histag protein was detected through western blotting (Figure 3.16). Specific antibodies were used to detect the presence of protein in these samples. For Western, two transgenic samples which were PCR positive were used.

No band was observed for wild type whereas bands of expected size (61 kDa) were observed for transgenic samples. This confirmed the protein expression in these transformed *Solanum lycopersicum* L. plants.

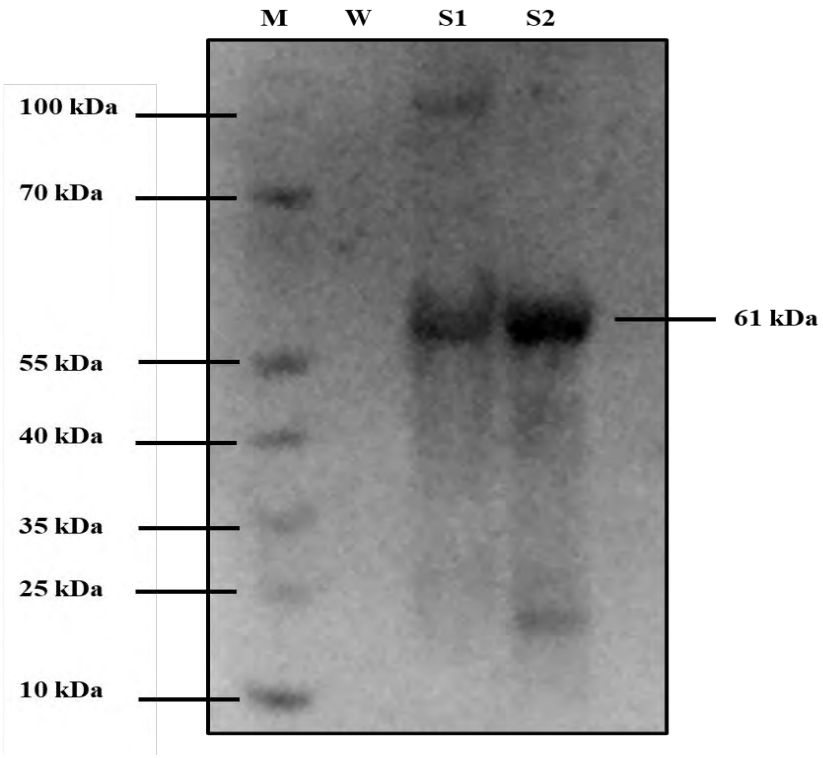

Figure 3.16: Confirmation of OmpK protein by Western blotting. (L). Ladder (W). Wild type (S1). Transformed sample 1 (S2). Transformed sample 2

3.5.5. ELISA

Indirect ELISA was used to confirm the presence of OmpK antigen within our transgenic samples. Total soluble protein extract from transgenic plants was used. As the TMB substrate was added, the blue color appeared in all samples except for blank and wild-type samples. The addition of 0.1N H_2SO_4 (stop solution) changed this blue color to yellow.

The data presented in Figure 3.17 shows an increased absorbance of transgenic samples having OmpK protein compared to the wild-type one. Among the two samples, S1 shows the highest absorbance which means that it contains the highest amount of antigen.

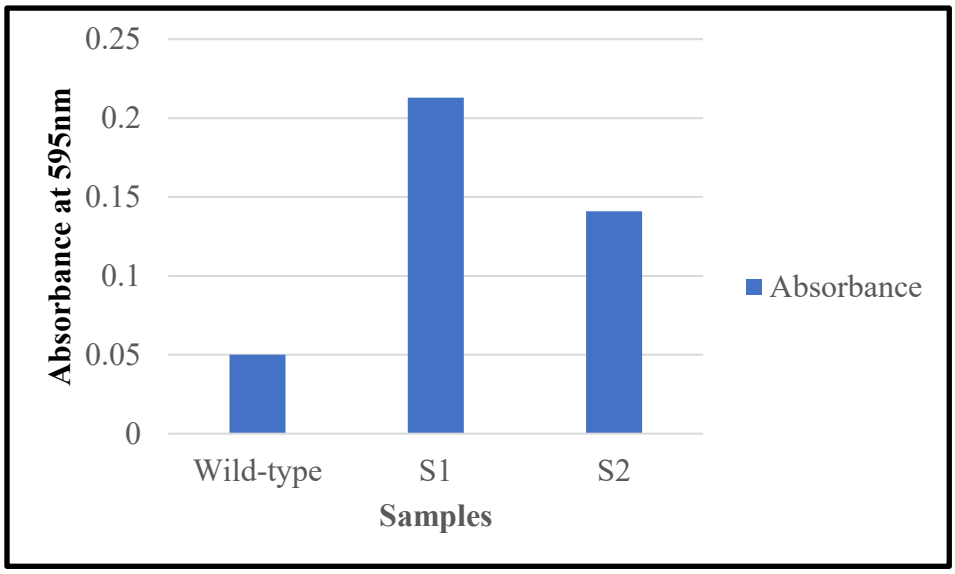

Figure 3.17: Graphical representation of ELISA of wild-type sample and transgenic tomato samples. S1 and S2 are two transgenic nodal samples

DISCUSSION

Vibriosis, caused by *Vibrio anguillarum* is a fetal infection that affects many fish species. By inducing hemorrhagic septicemia, it leads to the death of a great number of fish species and hence causes economic losses as well (Irshath *et al*., 2023). It is one of the major infections that cause the death of fish species on a large scale in the aquaculture industry which need to be controlled to prevent losses.

Despite the possibility of using antibiotics or chemotherapeutics to treat fish diseases, these methods come with several evident drawbacks, including the development of drug resistance and environmental and consumer safety issues. Vaccination is an effective strategy for preventing a wide range of bacterial and viral illnesses and helps to ensure the worldwide viability of aquaculture production (Muktar *et al*., 2016). Different kinds of fish vaccines have been made so far against vibriosis such as live attenuated vaccines, inactivated vaccines, and subunit vaccines (Su & Su, 2018). There are safety concerns associated with the live vaccines and the risk of virulence reversion. Inactivated vaccines are costly and need a high amount of antigens. However, recombinant subunit vaccine has potential advantages as they are easy to control and manufacture, and has high stability, and safety (Su *et al*., 2021). Subunit vaccines against fish vibriosis have been tried using different proteins of *Vibrio anguillarum* such as outer membrane proteins and flagellin proteins. Recent studies have shown that several vaccines based on the recombinant outer membrane proteins (including OmpK) of *V. anguillarum* provide effective and long-lasting immunity (Zhou *et al*., 2018; Xing *et al*., 2020; Li *et al*., 2022). These proteins can be genetically engineered in plants. The plant-based vaccines have advantages over these as they are cost-effective, safe, easily scalable, and can be made on a large scale. Moreover, it can be administered orally. Oral vaccination is considered the most ideal method of administration of vaccines to fish species (Embregts & Forlenza, 2016).

In the current study, the *OmpK* gene from *Vibrio anguillarum* is stably transformed in *Solanum lycopersicum* L. via *Agrobacterium*-mediated transformation to develop a plant-based edible vaccine against fish vibriosis.

4.1. OmpK as a potential vaccine candidate

According to the literature, OmpK is shown as a protective antigen against vibriosis. In 2012, researchers effectively generated a subunit vaccine with the OmpK

protein from *V. anguillarum* after cloning its gene in *Escherichia coli*. Animal studies have shown that this subunit vaccine has a good protective effect, with a relative percent survival (RPS) rate value of 67.8% (Hamod *et al*., 2012). According to Xing *et al*. (2017), 62.16% RPS is shown using recombinant OmpK against *V. anguillarum* in flounder fish species. Xu *et al*. (2018) demonstrated the cellular immune and humoral response in fish and the production of IgM against *V. anguillarum* when injected with the OmpK vaccine and obtained an RPS rate of 50% against *V. anguillarum.* According to Xing *et al*. (2020), the recombinant OmpK vaccine against *V. anguillarum* has induced T and B lymphocyte response in flounder and produced antibodies as well. In another study, an RPS of up to 70% is obtained using a bicistronic DNA vaccine of OmpK (Li *et al*., 2022).

4.2. Use of Tomato (*Solanum lycopersicum* L.) as a model plant

Tomato (*Solanum lycopersicum* L.) is a nutritive and important crop in the world and for genetic engineering purposes, it is regarded as a model plant due to several characteristics such as compact and small size genome, short life cycle, and self-fertilizing ability (Chetty *et al*., 2013).

4.3. Tomato seeds sterilization and germination

For seedling generation in a tissue culture experiment, surface sterilization of seeds is an important requirement to reduce contamination. In the current study, before transformation, surface sterilization and germination of tomato seeds cv. Rio Grande was done using 0.1% mercuric chloride ($HgCl_2$) for 15-20 seconds on half MS media and it produced the germination efficiency of 80%. The rate of germination was affected by the time of exposure to mercuric chloride which may be due to the bleaching action of mercuric chloride. Previously in a study three sterilizing agents i.e., mercuric chloride, sodium hypochlorite, and hydrogen peroxide were used to surface sterilize the tomato seeds and it resulted that the 5% sodium hypochlorite for 15 minutes gave the highest germination efficiency (Himabindu *et al*., 2012). In another study, 0.1% mercuric chloride is used for 8-10 minutes for surface sterilization of tomato seeds (Kumar *et al*., 2017).

4.4. Tomato plant regeneration

The kind and concentration of phytohormones for the regeneration of tomato explants is a crucial key element that regulates the shoot and root induction. In this study,

2mg/L zeatin was used as a regeneration hormone along with full MS with vitamin media for the tomato nodal explants regeneration. As a result, 100% callogenesis efficiency and 73.33% shoot regeneration efficiency were seen. Moreover, rooting was also observed on the same media after 30 days. Zeatin was found to be more effective than BAP for tomato explants. According to a study, nodal explants of tomato cv. Rio Grande showed the highest transformation efficiency (Hashmi *et al*., 2022). Previously in a study zeatin was used as a regeneration phytohormone for hypocotyl and cotyledonary explants of tomato (Pawar *et al*., 2012).

4.5. Hygromycin selection

In the present study, the concentration of hygromycin was optimized for checking the lethal dose of untransformed tomato explants and the selection of transformed tomato explants after infection with *Agrobacterium* having *OmpK*. Different concentrations of hygromycin including 0, 10, 15, 20, and 25 mg/L were added to the regeneration media of tomato nodal explants, and it was observed that no callus and shoot regeneration occur in the case of 25mg/L and this concentration was used in the current study. A similar lethal dose was used in another study and hygromycin was regarded as an effective selective agent for the selection of transformed plant of tomato cv. Rio Grande (Chaudhry & Rashid, 2010).

4.6. Stable *Agrobacterium*-mediated transformation

For the efficient stable *Agrobacterium*-mediated transformation, many factors are involved that influence its efficiency. These include the density of bacterial inoculum, infection time, and co-cultivation time. The selection of OD is a crucial factor that determines the transformation efficiency. In the current study, 0.6 to 0.8 OD_{600} was found to be effective for transformation. OD_{600} higher than 0.8 has an adverse effect on the explants and it also decreases the infection efficiency of *Agrobacterium*. 0.6 OD_{600} was observed to be the best for the stable transformation of tomatoes with *Agrobacterium* having *OmpK*.

Infection time and co-cultivation time for *Solanum lycopersicum* L. were also optimized in the current study. Two different infection times were observed i.e., 8-10 minutes and 15 minutes. The explants that were infected for 8-10 minutes regenerated more with 60% regeneration efficiency while those infected for 15 minutes showed less regeneration with 20% efficiency. So, the 8-10 minutes time period was observed

to be the best infection time for the transformation of tomato cv. Rio Grande with *Agrobacterium*. 10 minutes was also stated as ideal in another study by Wu *et al*. (2006).

Co-cultivation time was also evaluated by cocultivation of tomato explants after infection with *Agrobacterium* having *OmpK* for 2 and 3 days. The explants with 2 days of co-cultivation resulted in higher transformation or regeneration efficiency than 3 days. 2 days of co-cultivation was the best for stable transformation of tomato. The same results were shown by Jabeen *et al*. (2009). It was seen that with the increase in both the co-cultivation and infection time, the bacteria were grown too much which showed adverse effects on the plant growth. Moreover, the use of acetosyringone enhanced the transformation efficiency. 200µM acetosyringone was added to the co-cultivation and infection media for this purpose. The same concentration was used in another study by Chaudhry & Rashid. (2010). To control bacterial growth, cefotaxime was regarded as the best antibiotic for *Agrobacterium* (Sun *et al*., 2015). 500mg/l cefotaxime in the washing media and 250mg/l in the selection media was used.

Preculture of the explants on the regeneration media for one or more days before the infection and co-cultivation has been shown to be important as it increases the transformation efficiency. Devi *et al*. (2012) have shown that the preculture for one day appeared to be important which increased the regeneration efficiency of explants as well as the expression of transgene in tomato. Moreover, in some studies, explants were placed in the preselection media for several days after infection and washing before shifting them to hygromycin selection plates. Pre-selection media consisted of regeneration media supplemented with cefotaxime. Jabeen *et al*. (2009) stated that the placing of explants of tomato cv. Rio Grande on pre-selection for seven days increased the transformation efficiency.

For the confirmation of the integration of the transgene and its expression, various molecular techniques were used. These include PCR, Dot Blot analysis, qRT-PCR, Western Blot, and ELISA. For stable integration of transgene in transformed tomato explants, PCR was performed using specific primers. Wild-type explants were used as control. The presence of 890bp PCR fragments with *OmpK* nuclear primers and 500bp PCR fragments with *OmpK* internal primers confirmed the presence of the *OmpK* gene in the tomato-transformed explants. No bands occurred in the wild type.

Using the Wen *et al*. (2012) methodology, qRT-PCR was carried out to determine the copy number. Positive results were shown in transgenic samples and no results were shown in wildtype. The copy number for the *OmpK* gene was equal to 2.

For the analysis of OmpK protein, Dot blot, and Western Blot was done. In the Dot blot, the Chemiluminescence signal was shown in the total soluble protein extracted from transgenic samples i.e., sample 1 and sample 2 while no signal was seen in the case of wildtype.

Similarly, in Western Blot, after extracting the total soluble protein, PAGE was run, and the bands were transferred to the nitrocellulose membrane. After treating the blot with primary and secondary antibodies, and then with the substrate, visualization of the blot is done. A band of 61kDa was seen in both stably transformed protein samples i.e., in S1 and S2, and no band was shown in wild type. 61kDa band confirmed the presence of OmpK protein (dimeric form) in the transgenic samples of tomato plants.

Further, ELISA was also performed to confirm the OmpK protein. The presence of the OmpK protein in transgenic protein samples was confirmed when the TMB substrate was added, and the color change occurred. The absorbance at 595 nm was taken which showed that the S1 transgenic sample has a greater amount of protein than the S2 sample.

4.7. Conclusion and future perspectives

In this study, we have tested and optimized different variables required for the stable *Agrobacterium*-mediated transformation of *Solanum lycopersicum* L.

- Tomato cv. Rio Grande seeds could be sterilized by treating them with 0.1% $HgCl_2$ for 15-20 seconds and can be germinated efficiently on half MS media.
- Tomato cv. Rio Grande nodal explants can be regenerated with full MS with vitamins supplemented with 2mg/l zeatin.
- Factors like hygromycin concentration for selection, infection time, co-cultivation time, and bacterial density were optimized.

The current study provides optimized conditions for the stable transformation of *Solanum lycopersicum* L. cv. Rio Grande with *OmpK*. Maximum expression of transgene was observed when nodal explants of tomato were co-cultivated for 2 days

after 10 minutes of infection time. Taken together, in the future, this data could facilitate the development of a cost-effective antigen-based subunit vaccine against fish vibriosis, which may have the potential to be delivered orally. Also, this research can help in the development of an effective procedure for stable plant *Agrobacterium*-mediated transformation in various tomato cultivars using suggested optimum conditions for different variables.

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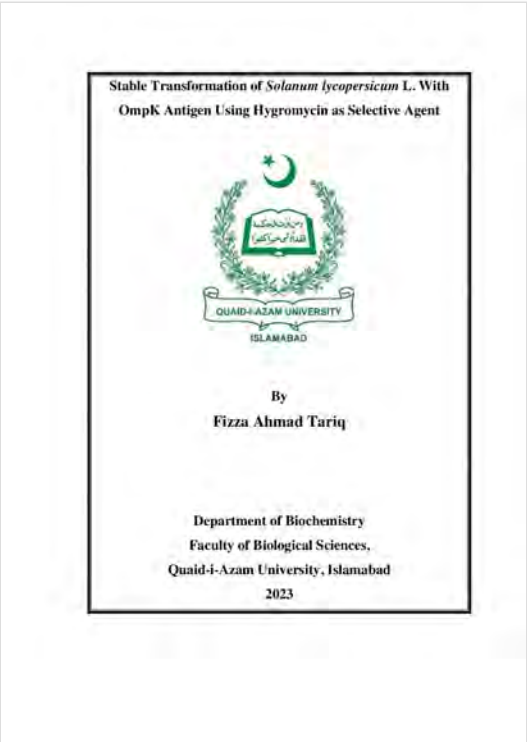

Stable Transformation of *Solanum lycopersicum* L. With OmpK Antigen Using Hygromycin as Selective Agent

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