Stable expression of Human Papillomavirus (HPV) Type 18 *L1* antigen in Tomato

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CERTIFICATE

This thesis, submitted by **Ms. Arooba** 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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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 Plant Biotechnology Lab, Department of Biochemistry, Quaid-i-Azam University, Islamabad.

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

Arooba

This thesis is whole-heartedly dedicated to my ***loving parents*** for their unconditional love, support, and courage

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LIST OF ABBREVIATIONS

A	Alpha
B	Beta
BSA	Bovine Serum Albumin
Bp	Base pair
°C	Degree centigrade
Cm	Centimeter
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo-nucleicacid
Da	Daltons
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
HPV-18	Human Papillomavirus Type 18
F	Forward
FAO	Food and agriculture organization
G	Gram
ϒ	Gamma
HEPA	High Efficiency Particulate Absorption
HCl	Hydrochloric acid
HRP	Horse-radish peroxidase
IAA	Indole acetic acid
IBA	Indole butyric acid
ICBN	International Code of Botanical Nomenclature
LB	Luria Broth
LA	Luria Agar
LDL	Low-density lipoprotein
LFH	Laminar Flow Hood
Mb	Mega bases
mM	Millimole

Mg^{+2}	Magnesium ion
mg	Milligram
mg/ml	Milligram/milliliter
mg/mol	Milligram/mole
MS	Murashige and Skoog
N	Normal
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
Ng	Nanograms
NLS	Nuclear localization signal
O.D	Optical Density
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
Rpm	Revolutions per minute
SQend	Starting quantity of endogenous gene
SQtrans	Starting quantity of transgene
TSP	Total soluble protein
T-DNA	Transfer deoxy ribo nucleic acid
Ti	Tumor Inducing
T4SS	Type IV secretion system
TMB	3,3',5,5'-Tetramethylbenzidine
μl	Micro liter
μm	Micrometer

UV	Ultra-violet
VLP	Virus like particles

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Finally, I would like to thank everybody important to the successful completion of this thesis, as well as expressing my apology that I could not mention them personally one by one.

AROOBA

ABSTRACT

Cervical cancer, attributed to human papillomavirus (HPV) infection, ranks fourth among women-related cancers globally. Predominantly prevalent in low to middle-income countries, cervical cancer poses a significant health challenge due to limited access to vaccination. Existing vaccines are costly, necessitate cold chain maintenance, and demand professional administration. Consequently, the imperative for a cost-effective and robust cervical cancer vaccine is evident. The present study focuses on utilizing HPV-18 from the Human Papillomavirus as a prospective vaccine candidate. Employing plants as an expression system for subunit vaccines is advantageous, and this research aims to establish an efficient and stable *Agrobacterium*-mediated transformation protocol for *Solanum lycopersicum* L. cv. Rio Grande to express the HPV-18 *L1* antigen. The investigation encompassed optimizing conditions for seed sterilization, germination, and regeneration of tomato explants. Promising outcomes were achieved with seeds germinated on half MS media following sterilization with 0.1% mercuric chloride. Zeatin at a concentration of 2mg/L demonstrated favorable regeneration efficiency. Nodal explants of *Solanum lycopersicum* L. exhibited optimal growth on 75mg/L Kanamycin, the preferred concentration for transformed explant selection. Successful transformation with HPV-18 antigen was accomplished, with an infection time of 8 minutes and a co-cultivation period of 2 days yielding the highest transformation efficiency of 60%. PCR validation using gene-specific primers confirmed the success of the transformation. Transgenic tomato explants were subjected to quantitative real-time PCR (qRT-PCR) analysis, comparing transgene expression to the β-actin gene. Additionally, protein expression was verified through Dot blot, Western blot, and ELISA. In conclusion, the accomplished stable transformation of *Solanum lycopersicum* L. with HPV-18 holds promise for the development of an economically viable subunit vaccine against cervical cancer.

Keywords: *Solanum lycopersicum* L., HPV-18 *L1*, Cervical Cancer, LTB-L1, *Agrobacterium*-mediated transformation, qRT-PCR, Western blotting, ELISA

1. Introduction

1.1. Cancer

Cancer results from unchecked cell division metastasizing into nearby tissues or organs and producing malignant tumors. According to the World Health Organization (WHO), 9.6 million cancer-related deaths and 18.1 million new cases were reported globally in 2018, making cancer the second highest cause of death worldwide. The development of cancer is influenced by several intrinsic and extrinsic risk factors (Wu *et al*., 2018). Random errors in DNA replication are the intrinsic factors, but extrinsic factors include genetic, behavioral, and environmental factors as well as viral and bacterial infections that can result in benign and cancer-causing metastatic tumors (CRI Staff, 2020).

1.2. Cervical Cancer

Cervical cancer is the fourth most frequent cancer worldwide among women and ranks seventh overall (Ferlay *et al*., 2015). Annually, 0.3 million people die from cervical cancer, with 0.6 million instances being detected (Sanami *et al.,* 2021). In developing nations, cervical cancer is the second most frequently diagnosed cancer in women and the third greatest cause of cancer-related fatalities. Developing nations account for over 90% of cervical cancer cases as well as deaths that are associated with them (Torre *et al.,* 2015).

The cervix in the genital tract is the target of cervical cancer, which is a malignant tumor that develops from a persistent lesion (Park *et al.,* 1995). Squamous-cell carcinoma and Adenocarcinoma are the two other forms that are classified into it. Squamous-cell carcinoma is more common and is responsible for 80% of the histological cases of cervical cancer (Katanyoo *et al.,* 2012). Squamous-cell carcinoma begins in squamous cells that are present on the outer surface of the cervix, whereas Adenocarcinoma develops in the glandular cells of the cervical canal (Hull *et al.,* 2020).

Squamous-cell carcinoma has three stages (Cervical Intra-epithelial Neoplasia-CIN). The first stage is CIN-I, in which malignant cells replace one-third of the epithelial layer, and the second stage is CIN-II, in which cancerous cells replace half of the total epithelium thickness. Finally, the CIN-III stage has malignant cells across the

epithelium, with normal cells forming the basement membrane. When these abnormal cells infiltrate nearby tissues by disrupting the basement barrier, the cancer becomes aggressive (Juneja *et al.,* 2003). Figure 1.1 depicts differential stages of cervical cancer development.
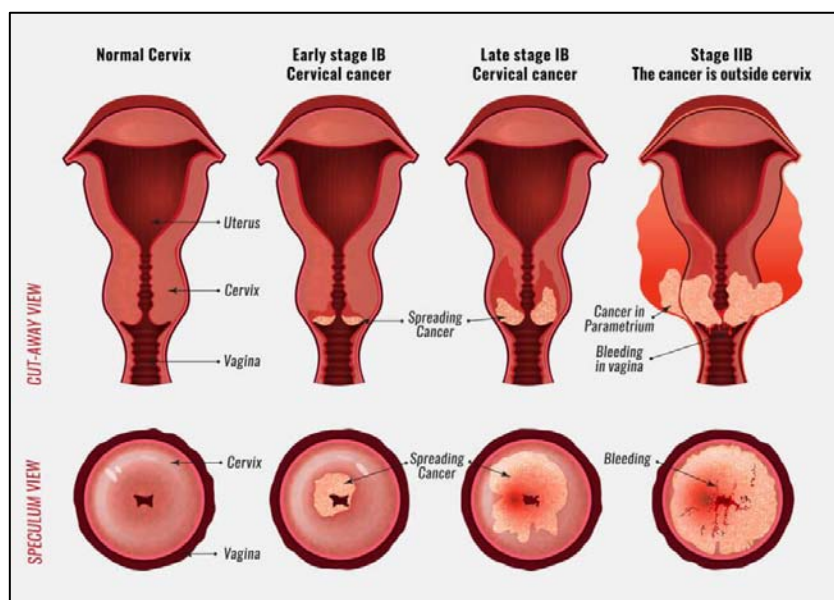
Figure 1.1: Comparison of Normal cervix with Cervical cancer development (https://singingriverhealthsystem.com/areas-of-care/cancer-care/womenscancers/cervical-cancer)

1.2.1 Prevalence of Cervical Cancer

Cervical cancer is more common in developing and underdeveloped countries. Lack of awareness, less screening for cervical cancer through Pap smear testing, and lower vaccine coverage are some of the causes for the increased prevalence of cervical cancer in low-income countries (Mwaka *et al.,* 2016). However, with widespread immunization and treatment of early cervical lesions in industrialized nations, total diagnosis and deaths have been reduced during the last four decades (Denny *et al*., 2017). Due to disparities in resource accessibility, developed nations exhibit lower mortality rates compared to low-income countries. Less developed regions experience a staggering 18-fold increase in the number of fatalities (Vu *et al*., 2018).

1.2.2 Cervical Cancer Risk factors

Cervical cancer is caused by a variety of significant and minor risk factors. The persistent infection of Human Papillomaviruses (HPV) is a major risk factor (Bosch *et al.,* 1995). HPV affects the muco-epithelial lining of the vaginal tracts and causes

lesions in the oropharyngeal area. HPV infects the cervix in girls as young as 22 years old, and in most cases, the infection resolves on its own; however, if the infection persists for years or decades, cervical cancer develops (Petry, 2014). Bad menstrual hygiene, early marriage, unprotected intercourse, and having intercourse with multiple partners are all risk factors for the development of cervical cancer (Kashyap *et al.,* 2019).

Immunocompromised people are more likely to acquire cervical cancer because their immune systems are unable to eliminate the HPV infection, which eventually leads to cancer growth. Women with a weakened immune system who are infected with the Human Immunodeficiency Virus (HIV) develop pre-invasive cervix lesions (Chirenje, 2005). Along with genetic abnormalities that cause cervical cancer, smoking (which contains nicotine, a carcinogen for cervix epithelial cells) is a risk factor for cervical cancer (Fonseca-Moutinho, 2011). Figure 1.2 depicts the probable risk factors for cervical cancer.
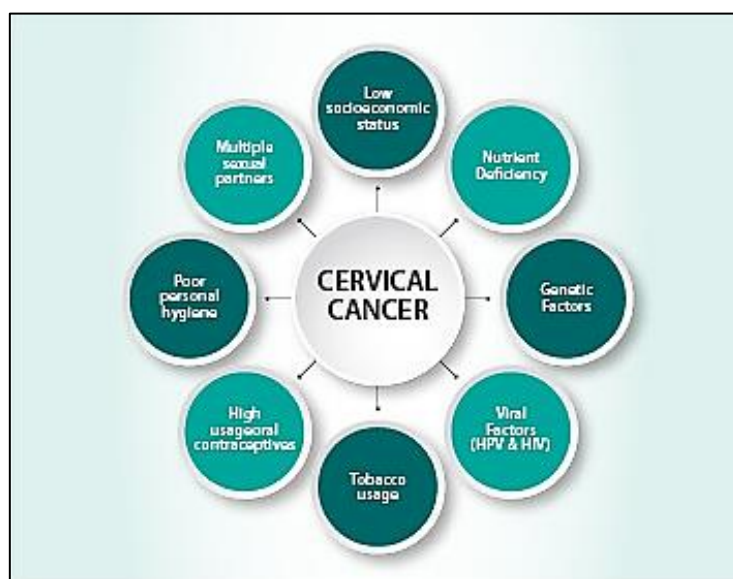
Figure 1.2: Risk factors for Cervical Cancer

1.3. Human Papillomavirus

The *Papillomaviridae* family of viruses includes HPV, a double-stranded DNA virus that is non-enveloped and has a stronger affinity for binding to epithelial cells. They are renowned for their capacity to result in infections of the cutaneous and mucosal epithelium. Over 207 different HPV types have been found; these are divided by genera such as α, β, γ, μ, and v.

The α HPV is the largest genus of viruses, known for infecting mucous membranes. 12 high risk HPV types—including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59—are responsible for around 90% of all instances of cervical cancer (Panahi *et al.,* 2018). The second-largest genus, β HPV, is mostly responsible for infecting cutaneous epithelia in immunocompromised people and those who have epidermodysplasia verruciformis disease-related genetic alterations (Pass *et al.*, 1977; Pfister, 2003). When paired with direct UV exposure, this HPV β also contributes to non-melanoma SCC (squamous cell carcinomas), one of the most frequent cancers in people (Tommasino, 2017; Van Doorslaer, 2013).

1.3.1. HPV Structure

The HPV genome is 8 kb in size. It has a Long Control Region (LCR), and the early region and late region are its two main regions. The two polyadenylations (pA) sites separate the LCR, early, and late regions. The sites are early pA and late pA. Proteins encoded by the early area are E1, E2, E4, E5, E6 and E7, whereas only two proteins are encoded by the late region, L1 and L2 (Zheng and Baker, 2006). While the late promoter in the E7 gene controls the late region, the early promoter, which is in the upstream regulatory region, controls the early region**.**

Most papillomaviruses have non-enveloped structural assemblies (Doorbar *et al.*, 2015). The L1 and L2 proteins combine to make the HPV capsid. The structural composition of the HPV virion is characterized by a T=7 icosahedral architecture, resulting in a 55nm particle. This assembly primarily involves 360 copies of the L1 major capsid protein and 12-72 copies of the L2 minor capsid proteins.

The L1 proteins form pentameric capsomeres, which further connect through disulfide bridges, ultimately constructing the capsid. The typical configuration includes 12 L2 molecules per capsid; however, varying reports suggest the potential for up to 72 L2 molecules (Conway *et al.,* 2009). The intricate interplay of L1 and L2 proteins in capsid assembly is essential for the overall structural integrity of the HPV virion, emphasizing the complexity and adaptability of this process. Natural HPV infection in terminally differentiated basal keratinocytes complicates laboratory maintenance. Traditional models like xenografts and raft cultures have been used, but they are costly and require specialized skills. Synthetic alternatives, such as VLPs and pseudovirions, produced in various cells, offer more accessible ways to study and

produce HPV particles. The structure of Human papillomavirus-18 is shown in Figure 1.3 below.
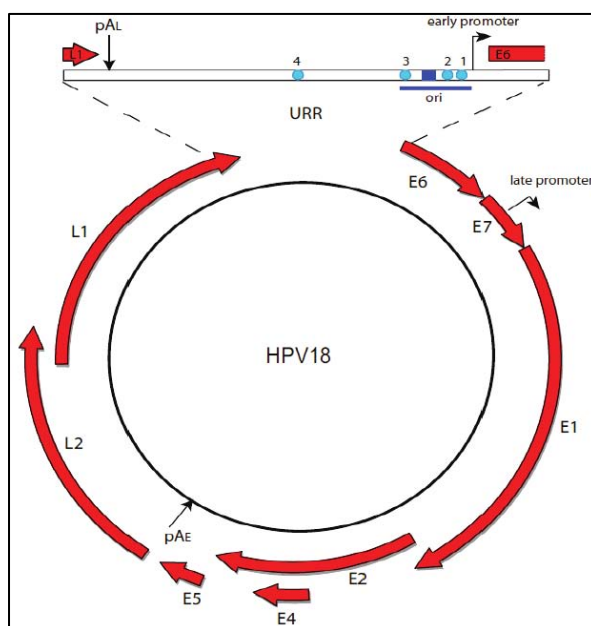
Figure 1.3: Structure of Human Papillomavirus-18

1.3.2. Human papillomavirus infectivity

Human Papillomavirus (HPV) gains entry into the host through microscopic abrasions in the skin, attaching itself to heparin sulfate proteoglycan receptors (HSPGs) present either on the skin's basement membrane or basal epithelial cells (Johnson *et al.,* 2009). This attachment triggers a series of events, including a cyclophilin-B-mediated change in capsid conformation, causing the L2 protein to undergo furin-mediated cleavage. This conformational shift in L1 enables its attachment to a secondary receptor on basal keratinocytes, initiating virus entry through a process known as clathrin-independent micropinocytosis (Kines *et al.,* 2009).

Subsequently, Cyclin B separates L1 from the L2/genome complex, directing it to lysosomal degradation. The L2 protein plays a crucial role in transporting the viral genome from the trans-Golgi network to the nucleus (Day *et al.,* 2008). However, nuclear entry of the viral genome only occurs during the mitosis phase. Once inside the nucleus, the L2/genome complex localizes at nuclear domain 10, facilitating transcription (Aksoy *et al.,* 2017).

It's noteworthy that most HPV infections are effectively cleared by the host's natural defenses (Schiller *et al.,* 2010). However, in cases where the immune system is

compromised, and there is infection with a high-risk (HR) HPV type, disease progression may occur. This complex interplay of viral entry, trafficking, and replication underscores the intricate mechanisms by which HPV establishes infection and highlights the importance of the host immune response in preventing disease development.

1.3.3. Vaccines targeting cervical cancer induced by the human papillomavirus

So far, three cervical cancer preventative vaccinations have been commercialized. L1 VLPs (virus-like particles) serve as the foundation for these vaccinations. In 2006, Gardasil® quadrivalent vaccine was the first to be marketed in. This vaccination is approved for use in both females and males and protects against 70% of cervical cancers and genital warts (Winer *et al*., 2006). Then, in 2007, GlaxoSmithKline (GSK) released Cervarix®, which protects against HPV-16 and 18 and is now exclusively authorized for use in women. Finally, in 2014, the Gardasil® nonvalent vaccine was introduced in the United States. It is approved for usage in both men and women. These vaccines are extremely effective, with HPV incidence reducing by 56% in the United States in the first four after vaccine administration (Markowitz *et al.,* 2013).

1.3.4. VLPs: Virus-like particles

VLPs are synthetic, non-replicating viral particles that self-assemble and lack viral DNA. They have been employed successfully in contemporary HPV vaccines, producing large titers of neutralizing antibodies (Schiller and Lowy, 2018). These L1 VLP-based vaccinations have been demonstrated to be safe and efficacious in clinical trials and follow-up investigations (Handisurya *et al.,* 2016; Schiller *et al.,* 2012). The success of these vaccines can be linked to several VLP-related properties (Mohsen *et al*., 2017).

The first reason is since VLPs (HPV=55nm) are small enough to permeate through lymph channel pores and circulate throughout the body. Second, the repetitive structure of the VLP is ideal for stimulating the immune response via pathogen-associated receptor recognition, as well as providing stable crosslinking to B cell receptors, which may explain the high levels of neutralizing antibodies induced by these vaccines (Bachmann *et al.,* 1997; Bachmann and Jennings, 2010). Furthermore, the innate immune system components have a multimeric structure, which facilitates

robust binding to the VLP and so increases the efficacy of opsonization by antigen-presenting cells (Gomes *et al.,* 2017), resulting in very effective VLP-based vaccines.

1.3.5. Drawbacks of VLP-based vaccines

While these available vaccines have had considerable success, they are unable to operate therapeutically and hence cannot be used to treat those who are already ill. They also provide only a limited level of cross-protection against nonvaccine strains. Furthermore, they are costly (Schiller and Müller, 2015; Petrosky *et al.,* 2015), making them less accessible to low-income nations with incidence and death rates ranging from 20% in Africa to 50% in Asia (Bray *et al.,* 2018). The production and distribution costs for both Cervarix and Gardasil are nearly identical, costing around $130 per vaccine dose, and the estimated expense for the three doses totals $390, which does not include the expense associated with the vaccine's injectable delivery (Waheed *et al*., 2012). Finally, all the mentioned vaccines are VLP-based, and VLPs are thermally unstable; thus, a constant cold chain maintenance is essential to preserve vaccine stability (Waheed *et a*l., 2011). Therefore, the production of second-generation cost-effective vaccines is a need.

1.3.6. Capsomeres-based vaccines as an alternative

Capsomeres are a component of the capsid, which is an outer shell formed of protein that protects the virus's genetic content. Capsomeres organize themselves naturally to form capsid. VLPs (virus-like particles) are used in all commercially available HPV vaccines (Schiller *et al*., 2012). Alternatively, preparations including subunit capsomere protein, on the other hand, have shown a promising ability to induce antigenic response (Rose *et al.,* 1998; Zhou *et al.,* 2014; Fligge *et al*., 2001b; Jagu *et al.,* 2010; Wu *et al.,* 2011). The L1 capsomeres, which are the fundamental component of the HPV capsid, are created by the pentamer assembly of L1 monomers. In general, capsomere-containing formulations seem to be less immunogenic than their VLP counterparts. Capsomeres are equally immunogenic, nevertheless, when combined with adjuvants such as MPL (Monophosphoryl Lipid A) (Thones *et al.,* 2008). Furthermore, capsomeres have demonstrated the ability to protect against viral infection in the COP (canine oral papillomavirus) animal model and have been shown to induce high titers of neutralizing antibodies and T-cell

responses when immunization was carried out through oral, intranasal, or subcutaneous routes (Dell *et al.,* 2006; Fligge *et al*., 2001a).

1.3.7. Human papillomavirus-18 *L1* major capsid protein

L1, the main capsid protein of the human papillomavirus, is a ~56.5 kDa protein with the ability to spontaneously self-assemble (Schiller and Lowy, 2012). Each of the 72 unique knobs that make up the virion body's outer core is composed of a pentameric L1 capsomer (Modis, 2002; Wolf *et al.,* 2010). The extended C-terminus of L1 forms the inter-L1 disulfide bonds between the adjacent capsomers. Accordingly, the stability of virion depends critically on these important disulfide connections (Buck *et al.,* 2005; Conway *et al.,* 2009). L1 interacts with the heparin sulfate carbohydrates that are displayed on the outside of proteoglycans to facilitate most of the papillomavirus's host engagement (Giroglou *et al.,* 2001; Johnson *et al.,* 2008).

1.3.8. *Escherichia coli* heat-labile enterotoxin subunit B (LTB) as an adjuvant

The word adjuvant is derived from the Latin word *adjuvare,* which means "to help" (Cox and Coulter, 1999). In general, an adjuvant is an agent that, when combined or blended with a vaccine, enhances its immunogenic qualities. The right adjuvant choice is crucial for promoting robust humoral, or Abs-mediated and cellular immunity. The trials of Gaston Ramon, a scientist from the Pasteur Institute who first introduced the term adjuvant while working on the vaccines for horses, are where the history of adjuvant development begins. The alum-based adjuvanted vaccines have taken over the vaccine market ever since Glenny and his colleagues identified aluminium salts with adjuvant qualities in 1924 (Coffman *et al.,* 2010). Based on their mode of action, a variety of natural and synthetic compounds and biological molecules having adjuvant qualities are used. Adjuvants include mineral salts, microparticles, cytokines, and tiny liposomes (Guy, 2007; Awate *et al*., 2013; Reed *et al.,* 2013). Adjuvants can boost vaccine activity in a variety of ways including an increase in the natural half-life of vaccines, an increase in the natural interaction with antigen-presenting cells (APCs) such as dendritic cells, assistance in activating APCs, induction of immunoregulatory cytokines production, activation of inflammasomes and local inflammation, and induction of cellular recruitment (Nakaya *et al.,* 2016).

LTB, a known enterotoxin, is implicated in increasing enteric pathogen fidelity and aiding pathogen colonization in the small intestine. LTB is also a well-studied adjuvant that has been shown to increase the antigenicity of vaccinations. It can also be used as a natural cell targeting and antigen delivery approach, and it personifies novel platforms for enhanced vaccine development (Duan *et al.,* 2019). When combined with or fused to antigens, the LTB component has been exploited for its capacity to function as an adjuvant. According to reports, combining vaccine candidates with the GM1-binding LTB pentamer aids in the antigen's ability to target the mucosal tract precisely. For instance, the mucosal immune response in mice and dogs was enhanced when the LTB component was fused with rabies virus-like particles (Qi *et al.,* 2015). Like this, when *Mycoplasma hyopneumoniae* R1, P42, and NrdF antigens were fused to the LTB, mice and pigs had an improved immune response (Marchioro *et al.,* 2014). All these investigations demonstrated LTB's potent mucosal adjuvant properties when combined with or fused with a variety of antigens.

1.4. Plant Biotechnology

Biotechnology is defined as the use of living organisms and the manufacture of their products to enhance the living conditions of humans. The field has evolved from producing everyday goods like bread and beer to being a tool for creating customized species of plants and animals as well as producing human drugs for treatment (Jha and Ghosha, 2006). Modern biotechnology follows the classical biotechnology era and is characterized by innovative genetic modifications in animal and plant cells. Recombinant DNA technology and gene splicing, silencing, and activation are the fundamental components of the genetic modifications. Therefore, according to the contemporary definition, biotechnology is a group of methods for modifying genetic material, biological components, and cells to function as desired. The development of therapeutic drugs and the area of medicine have benefited from advances in biotechnology (Chawla, 2000).

Ancient plant biotechnology was exclusively motivated by the desire for novel plant breeds and domesticated crops on the part of humans (Jha and Ghosh, 2005; James, 2007). Modern plant biotechnology can be characterized as the collection of specific scientific methods for creating plant types that are both broadly helpful and useful as medicines. The development of plant cell culture, enhancement of tissue and organ

culture, and in vitro plantlet regeneration have cleared the way for new breakthroughs in plant biotechnology (Bhatia, 2019).

1.5. Plant tissue culture

Plant tissue culture is the aseptic and in vitro generation of cells, tissue, organs, and their component parts under controlled physical and chemical conditions (Thorpe, 2006). The man known as the "father of tissue culture," Haberlandt, carried out studies on single-cell culture and was the first to provide a theoretical justification for tissue culture (Thorpe, 2007). Tomato roots were the first plant parts to be effectively tissue grown (White 1934), followed by tobacco and carrot callus (Gautheret, 1934; Nob'ecourt, 1939), as well as highly dividing shoots and meristem cells (Ball, 1946). Tissue culture is utilized in large-scale plant production and multiplication. It is an important research tool. Furthermore, it has numerous industrial applications in plant propagation, secondary metabolite production, plant disease removal, and crop improvement (Hussain *et al.,* 2012).

1.5.1. Explants

Explants are tiny fragments of plants that can regenerate into a new complete plant. Explants might be plant leaves, stems, roots, or flowers (Idowu *et al.,* 2009). In tissue culture, we use several forms of explants, such as cotyledonary nodes or hypocotyl segments, and provide them with optimal environmental conditions as well as the right concentration of nutrients and hormones.

1.5.2. Culture media for plant

When a plant is grown in vivo, the essential nutrients and elements vital for its growth are supplied by the soil or fertilizers. Similarly, plant growth in tissue culture necessitates a range of macro- and micronutrients to support its growth, metabolism, and overall development. Tissue culture systems provide the necessary nutrients, energy, and water required for the growth of the plant or explant through basal media. The incorporation of phytohormones, whether natural plant growth regulators or their synthetic counterparts, at different stages of plant growth or development has a beneficial impact on the maturation of the plant. For this reason, various forms of basal media have been utilized (Phillips & Garda, 2019). MS media is the most extensively used media for plant tissue culture (Vasil, 2008). It contains chelated iron,

myoinositol, and four other vitamins. Other media for plant regeneration include BABI or B5, Schenk and Hildebrandt medium (SH medium), WPM, and DKW (Phillips & Garda, 2019).

1.6. Plant Biopharming

Biopharming is defined as the manufacture of biological medicines outside the body using living systems as hosts. When compared to other in-vitro systems, these living systems serve as biofactories to produce biological components that are either economically or technically impractical (LeBlanc *et al.,* 2020). *Escherichia coli* was used as the bacterial host in the first instance of this technique by Genentech in 1978, and it was later commercialized in 1982 (Quianzon & Cheikh, 2012). Following that, eukaryotic cells are also used as hosts, such as when CHO (Chinese hamster ovary) cells are used to manufacture anticoagulants and monoclonal antibodies (Jayapal *et al.,* 2007; Reinhart *et al.*, 2015). Currently, yeast, mammalian, avian, insect, plant, and bacterial expression systems are used in biopharming (Legastelois *et al.,* 2017). In comparison to other systems, using plants as hosts to create biological materials provides benefits. Plant-based systems are versatile and less expensive for the amplification of biomass than fermenter-based systems, which need a lot of time and money (LeBlanc *et al.,* 2020). The creation of synthetic human growth hormone from tobacco and sunflower is the first instance of biopharming in plants (Barta *et al.*, 1986). Tobacco, maize, lettuce, barley, tomato, rice, broccoli, pitcher plants, carrot, and soybean are only a few of the many additional plants that have undergone transformation (Kim *et al.,* 2012; Miguel *et al.,* 2019).

1.7. Plant-based edible vaccines

Vaccines help to revitalize antibody production in humans and animals, as well as increase immunity against infectious diseases (Doshi *et al.,* 2013). However, numerous vaccines for the treatment of deadly diseases are inaccessible in low-income countries due to their high cost and the inability to maintain a steady cold chain. This has focused global attention on the development of vaccines that are less expensive, safer, and easier to use.

Plant-based vaccines are cost-effective and have several other advantages over other approaches in this regard (Lössl & Waheed, 2011). These vaccines are created by genetically engineering the expression of genes encoding disease-specific antigens

into plant hosts. It can be accomplished through direct gene bombardment into plants or through *Agrobacterium*-mediated transformation (Kurup, 2020). They have demonstrated their efficacy in safeguarding against infectious diseases, autoimmune disorders, birth control, and cancer treatment. Genetically modified plants are now widely accepted in both industrialized and developing nations. Barley, tobacco, tomato, banana, lettuce, rice, wheat, maize, spinach, alfalfa, soybean, papaya, and cucumber have been utilized to produce edible vaccines.

The method of producing an edible plant-based vaccine that elicits an immune response is demonstrated in Figure 1.4. Plant-based vaccines have gained popularity because they are needle-free, quick, and easy to administer, do not require elaborate storage, and are cost-effective because large-scale manufacture is possible.
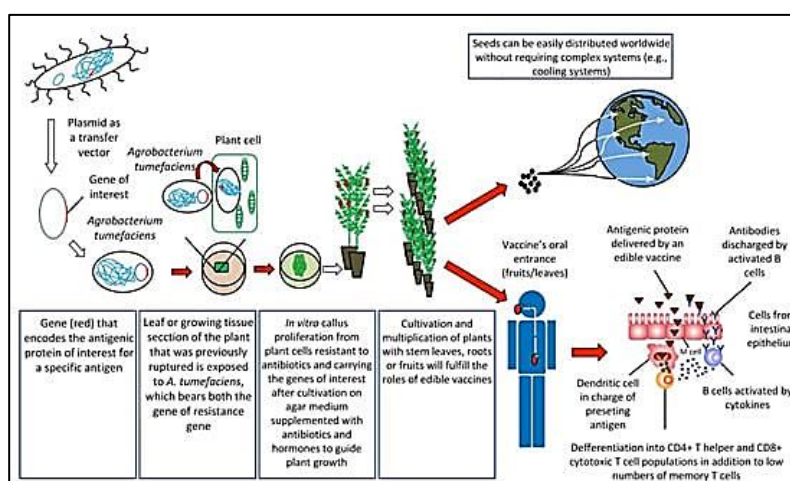
Figure 1.4: Method of producing plant-based edible vaccines and its immune response (Concha *et al.,* 2017)

1.7.1. Advantages of plant-based edible vaccines

The main advantage of using plant-based systems is their low cost. Plants can be grown on-site and scaled up to meet demand, as opposed to the costly instruments and infrastructure of time-consuming fermenter-based systems (Waheed *et al.,* 2016). Because antigens are stable in plant systems, there is no cost for maintaining the cooling chain, particular shipping, and storage. There is no risk of pyrogen or endotoxin contamination. As a result, severe purification and downstream processing are not required (Lössl & Waheed, 2011). They can be taken orally in raw form. Transgenic seeds may be easily preserved for a long time period at room temperature

and are also widely available as a source of vaccines. Furthermore, they have a strong cell wall that protects them from the acidic environment of the gut (Sack *et al.,* 2015).

1.7.2. Disadvantages of plant-based edible vaccines

Risks of plant-based vaccines include allergic reactions, hypertensive responses, and oral tolerance (Takeyama *et al.,* 2015). In this instance, proper dosage optimization is required. There is the possibility of inappropriate post-translational modifications. Another difficulty with plant-based expression is that it has the potential to infect non-transgenic plants in the field (Malabadi *et al*., 2015). However, because there is little chance of transgenic dissemination via pollen, this problem can be overcome by expressing antigens in chloroplasts (Waheed *et al.,* 2016).

1.8. Plant transformation

Plant transformation is defined as the insertion of a foreign gene of interest into the plant genome in order to produce a transgenic plant (Guo *et al.,* 2019). Researchers were able to manage the *Agrobacterium tumefaciens*, which causes the crown gall disease in plants, in the early 1980s to introduce foreign genetic material into plants, which is when the process got started (Newell, 2000). In 1984, tobacco was reported to have undergone the first plant transformation (De Block *et al*., 1984; Paszkowski *et al.,* 1984). In this method, a desired gene with a beneficial function is isolated, ligated with a suitable vector to produce recombinant DNA, and then the gene is transferred into the plant to create a transgenic plant. Desirable traits can be attained in this way. A crop's improved quality, higher yield, and self-resistance against diseases and pests can all be achieved with the help of genetic transformation (Sinclair *et al.,* 2004). The term "transgene" refers to the gene that will be transferred, and the resulting organisms are called 'transgenics'. Different strategies are being used to successfully transfer the foreign genetic material into the specific host organism. There are two types of strategies for gene transfer into plant genome.

• Indirect gene transfer

• Direct gene transfer

Agrobacterium-mediated gene transfer, agroinfiltration along with other vector-based techniques like viral-vector based delivery of genes for the transient as well as stable

protein expression (Chung *et al.,* 2006), is the most popular and well-studied indirect method of gene transfer (Franklin and Lakshmi, 2003).

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 are some of the direct ways that are currently accessible. Biolistic method is also referred to as gene gun or micro-projectile bombardment method. In this transformation strategy, gold or tungsten particles are coated with desired DNA or RNA. High pressure of helium is generated in the gene gun which is responsible for the penetration of DNA coated gold particles into the plant cells. Plant genetic transformation is very helpful in the development of disease-free, viral, or bacterial resistant plants. Methods of plant transformation is illustrated in figure 1.5.
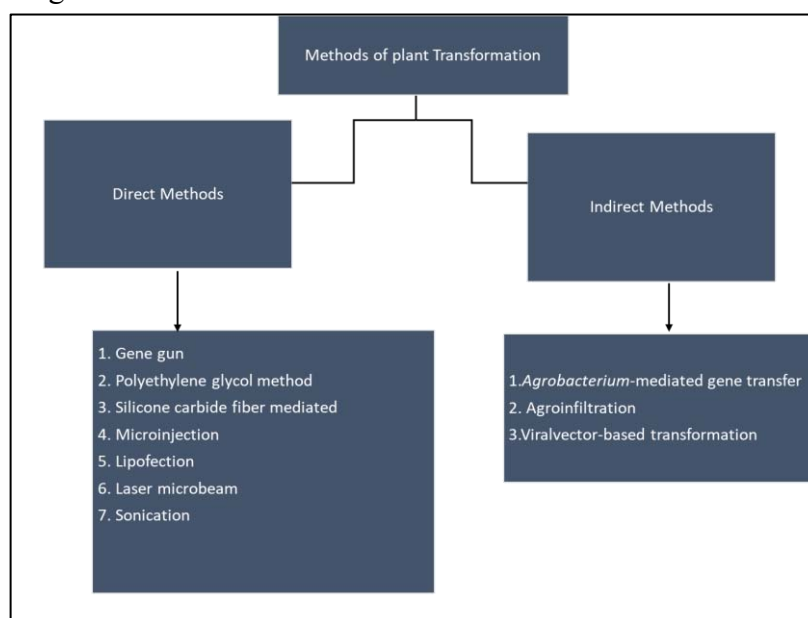
Figure 1.5: Strategies of plant transformation (modified from Saifi *et al.,* 2020)

1.8.1. *Agrobacterium*-mediated transformation

Agrobacterium is regarded as an effective method of introducing foreign genes into plants and a potent tool for introducing the desired gene into the cells of the host plant (Matveeva, 2018). The tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* has the capacity to transmit a specific DNA fragment known as T-DNA into the nucleus of infected cells, where it is integrated into the genetic structure of the host genome (Chilton *et al*., 1977).

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

There are two types of transformation done by *Agrobacterium*:

• Stable Nuclear Transformation

• Transient Transformation

The genetic material transferred during stable nuclear transformation is integrated into the host cell transmit into the next generation, whereas during transient transformation, the DNA is only present in the host cell for a brief period and is not passed on to the next generation (Hwang *et al.,* 2017).

1.8.1.1. Stable Nuclear transformation

Agrobacterium is commonly used for the stable nuclear transformation of foreign genes in plants. As a result, foreign genes are incorporated into the plant cell nucleus and passed on in a stable manner to succeeding generations (Horn *et al.,* 2004). Because of its uncomplicated process and lower cost compared to other approaches, *Agrobacterium* is typically used to transform plants.

With the use of this technique, larger segments—up to 150 kb—can be put into the genome of the plant. Therefore, a well-organized system of vectors was created for this purpose to successfully transfer genetic material into plants and to improve transformation efficiency (Hansen and Wright, 1999).

1.8.1.1.1. *Agrobacterium tumefaciens*

Crown gall disease in plants is mostly caused by the ubiquitous soil bacterium *Agrobacterium tumefaciens*, which belongs to the class *Alpha proteobacteria.* Crown gall is a type of plant tumor that can be caused by *Agrobacterium* due to the presence of a plasmid that induces tumors (Ti).

Ti has transferable DNA (T-DNA), a DNA that merges with the host plant cell nucleus to cause tumors or crown gall (Niazian *et al.,* 2017). Figure 1.6 shows the structure of *Agrobacterium tumefaciens.*
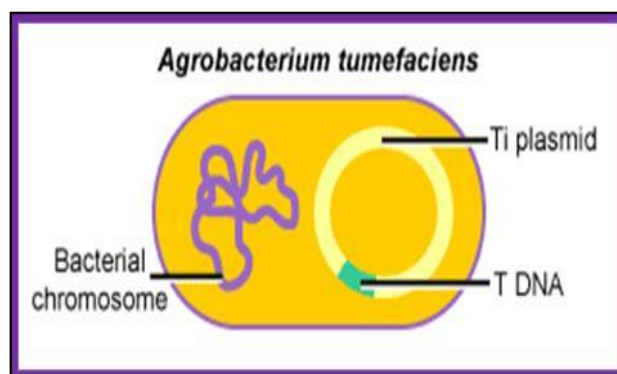
Figure 1.6: Structure of *Agrobacterium tumefaciens*

1.8.1.1.2. Ti plasmid

The approximately 200 kb Ti plasmid is typically present in pathogenic *A. tumefaciens* strains. The disease's information is carried by this plasmid. The Ti plasmid is divided into two distinct regions:

• T DNA

• Virulence region (*vir* region)

T-DNA is transferred to the host cell via the virulence region. It possesses distinct loci that hold the genetic information required for transmission. On the *vir* region are the following loci: *vir* A, *vir* B, *vir* C, *vir* D, *vir* E, *vir* F, and *vir* G. The expression of additional *vir* proteins that aid in further mediating the T DNA transfer process is facilitated by the Vir A and Vir G proteins. T-DNA processing and transport into host plant cells are aided by these proteins (Hwang *et al.,* 2017). It also contains the replication region's origin and genes involved in opine catabolism. Figure 1.7 shows the Ti plasmid diagrammatic representation.

1.8.1.1.3. T-DNA

T-DNA is surrounded by 25 bp segments as shown in figure 1.7 that are transmitted to host cells through *Agrobacterium*-mediated transformation. Once incorporated into the plant cell, T-DNA causes the synthesis of opines through the action of enzymes encoded in the DNA, which the bacteria need as nutrition to survive. T-DNA not only codes to produce opines but also for the enzymes needed to synthesize auxins and cytokinin in transgenic plants so they can regenerate (Zupan and Zambryski, 1995). T-DNA contains oncogenes, which are responsible for plant diseases. When plants are

infected with *Agrobacterium*, these oncogenes in the T-DNA can be replaced with our desired genes to achieve desired features (Hwang *et al.,* 2017).
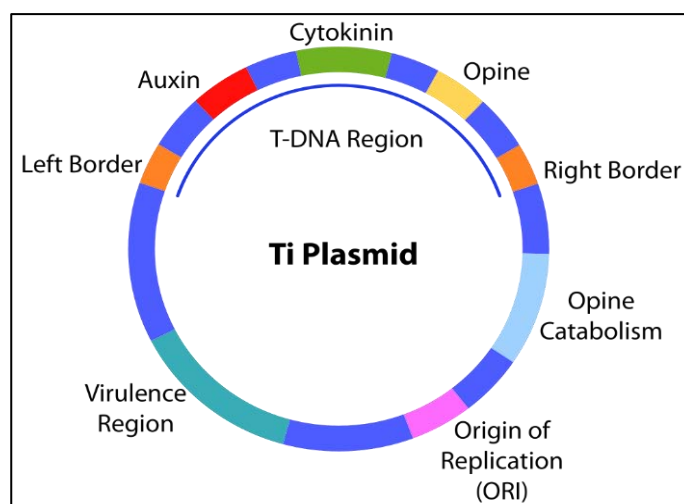
Figure 1.7: Diagrammatic representation of Ti plasmid (Alkuddsi *et al.,* 2014)

1.8.1.1.4. Steps of T-DNA integration

The delivery of T-DNA inside the host cell genome comprises different steps (Pratiwi and Surya, 2020)

1) Signal recognition through *Agrobacterium tumefaciens*

2) T-DNA processing

3) T-DNA migration into the host cell

4) Integration of T-DNA into the plant nuclear genome

5) Expression of inserted T-DNA in plant host cell

Signal recognition through *Agrobacterium tumefaciens*

The process begins when *Agrobacterium* detects plant signals. These include of low pH, low phosphate signals, sugars, and phenols (Brencic & Winans 2005). Certain *vir* genes are induced to express when bacteria recognize these signals (Pitzschke & Hirt, 2010).

Monocyclic phenols like acetosyringone are among the most potent signals (Stachel *et al.,* 1985). Several studies have suggested that this process begins at the plant's wound, where plants are infected by *Agrobacterium.* In addition to the site of entry, plants that have been injured generate an acidic sap that is rich in phenolic compounds

and aids in the chemotaxis of bacteria (Barampuram & Zhang, 2011). When bacteria detect these chemicals, the expression of the *vir* gene initiates.

T-DNA processing

Processing (cleavage) of the T-DNA from the Ti-plasmid is carried out by VirD1 and VirD2. The 25 bp border regions on the bottom strand of T-DNA serve as the excision site for VirD1 and VirD2. The helical DNA strands are unwound by the site-specific helicase VirD1, while the T-DNA is cut from both border sequences by VirD2. Consequently, T-strand, a single stranded DNA, is formed (Veluthambi *et al.,* 1987; Zambryski *et al.,* 1989).

While the 3' end of the T-strand serves as the primming site for the regeneration of the bottom strand of T-DNA, the 5' end of the T-strand is capped by VirD2 (Shimoda *et al.,* 1990; Zambryski *et al.,* 1989). VirC1 attaches to the right border sequence of T-DNA (Toro *et al.,* 1989). Finally, T-DNA is coated by virE2 forming. It functions to protect the T-strand from degrading inside the host plant cell (Barampuram & Zhang, 2011).

T-DNA migration into the host cell

The Vir D2/T-strand complex is moved into the host nucleus by the C-terminal nuclear localization signal (NLS), which is located on the VirD2 and VirE2 (Hwang *et al.,* 2017).

Integration of T-DNA into the plant nuclear genome

The integration of T-DNA into the nucleus of plant cells is the final stage of transformation performed by *Agrobacterium*. The incorporation of T-DNA within the plant cell is continued by VirD2. While VirE2 guards the left border of T-DNA, VirD2 likewise shields the right border from any kind of degradation (Hwang *et al.,* 2017).

Expression of inserted T-DNA in plant host cell

Once T-DNA is integrated into a plant cell's nuclear genome, it might follow two different paths. It is either integrated but not expressed at all, or it is expressed within the plant cell and expresses itself at different levels (Christie, 1997). The overview of this whole process is demonstrated in following figure 1.8.
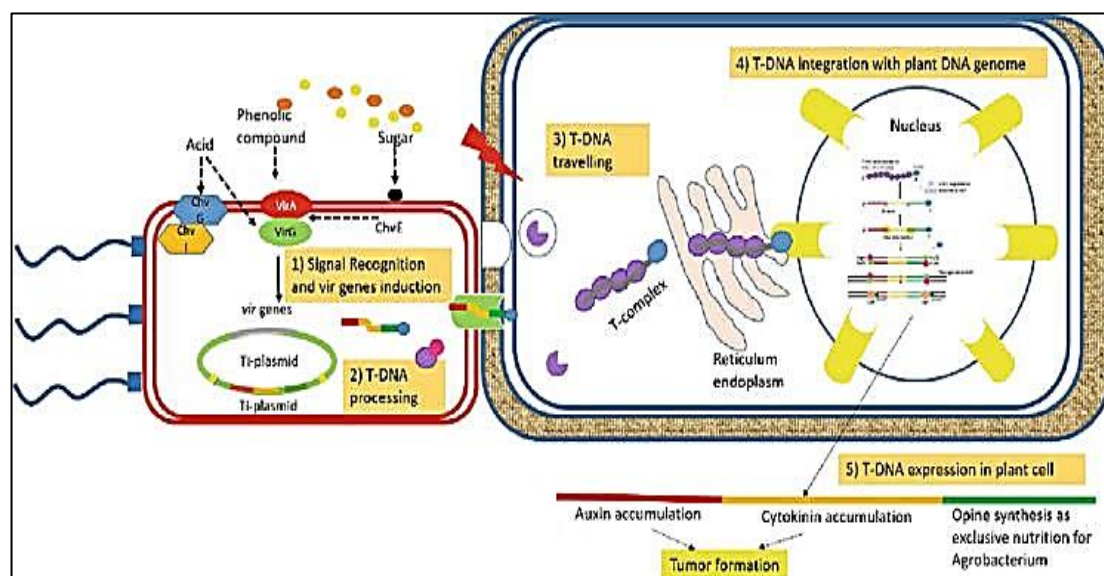
Figure 1.8: Overview of bacterial and plant interaction (To & K.-Y, 2020)

1.8.1.1.5. Mechanism of *Agrobacterium*-mediated transformation

The multiple steps that make up the stable *Agrobacterium*-mediated transformation are given below (Pratiwi and Surya, 2020):

1) Seed sterilization and preparation of inoculum

2) Selection and preparation of explant, infecting it with *Agrobacterium tumefaciens* and co-cultivation

3) Selection of transformed explants using optimized concentration(s) of antibiotic (s)

4) Regeneration of explants

5) Acclimatization and molecular identification

The success rate of stable transformation mediated by *Agrobacterium* is dependent on multiple parameters. These include of the plant's cultivar, the kind of explant used, the density of the bacterial suspension, the infection period, the co-cultivation period, the appropriate acetosyringone concentration, and the type and quantity of selection antibiotic (Asande *et al.,* 2020). The overview of the whole mechanism of transformation is shown in figure 1.9.
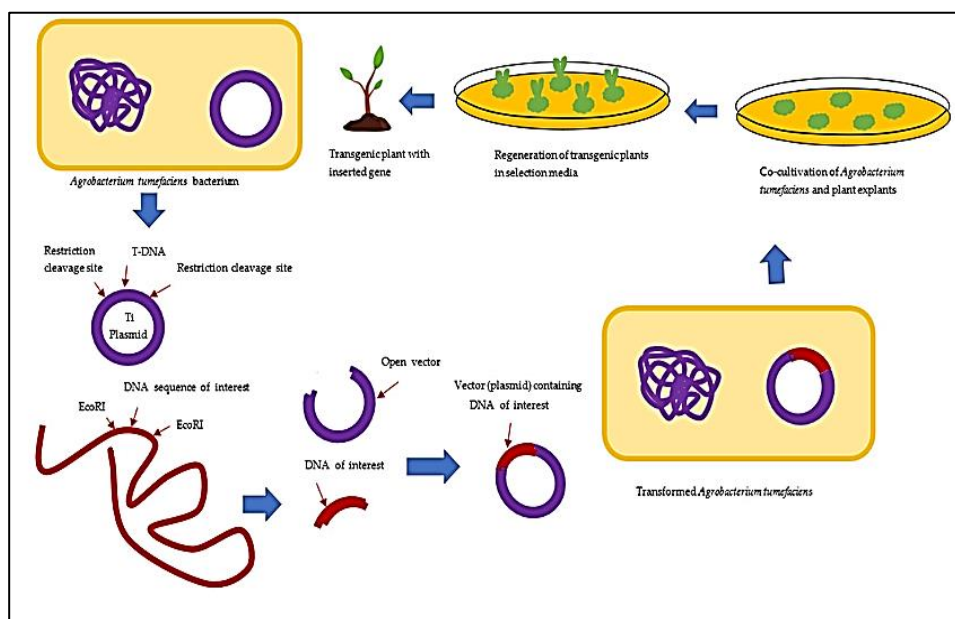
Figure 1.9: Mechanism of *Agrobacterium*-mediated transformation (Ghimire *et al.,* 2023)

1.8.1.1.6. Pros and cons of Stable Nuclear Transformation

The main advantages of steady *Agrobacterium* nuclear transformation are its high effectiveness in the transformation of plant cells and constant expression of the protein product of the gene of interest (Laere *et al.*, 2016). In comparison to alternative ways, the procedure is economical (Saba *et al.*, 2019). When compared to transient transformation, the drawback of stable nuclear *Agrobacterium* transformation is its longer processing period (Waheed *et al.*, 2016). Furthermore, the positional effect could be the outcome of random integration. Stable nuclear transformation may lead to transgene contamination and gene silencing (Fahad *et al.,* 2015).

1.8.2. Transient plant transformation

Since the stable transformation approach takes a long time to complete, a faster alternative strategy was created to save both time and resources. Due to many breakthroughs, the technique of transient transformation of plants has become relevant. Genetic material is transferred to the plant host cell using *Agrobacterium tumefacien* as a tool. To transport T-DNA from the trans-acting virulence proteins to the host cell, a binary system of vectors is employed. The target gene is transiently present in the host cell's somatic nucleus and is not passed on to the progeny.

Within 12 hours of infection, the target gene starts to express itself in the host cell, reaching a high expression level in two to four days (Sainsbury and Lomonossoff, 2014). The DNA that has been transferred does not combine with the host nucleus. It simply stays in the nucleus and expresses itself momentarily before losing its strength over time. The same procedure must be carried out again for the gene to express itself again (Kapila *et al.*, 1996).

Various techniques such as the biolistic method, polyethylene glycol (PEG) mediated transformation, electroporation, and agroinfiltration employing *Agrobacterium* can be employed for the transient transformation of the target gene (Wang & Jiang, 2011; Tsuda *et al.*, 2012; Tan *et al.*, 2013).

1.8.2.1. Agroinfiltration

Agrobacterium infiltration is an effective strategy because of its fast and efficient DNA transfer mechanism. This method involves infiltrating plant tissues with a suspension culture of agrobacterial cells containing our gene of interest, which results in the transient expression of the desired genes. Various techniques for agroinfiltration have been presented.

Among these are vacuum infiltration and syringe infiltration (agroinjection). Since vacuum infiltration produces poor expression of transiently expressed genes, it is not a trustworthy approach. Therefore, for the temporary expression of genes, syringe infiltration is the most dependable agroinfiltration technique. When using this strategy, one leaf is sufficient to bring about simultaneous collective transient expression experiments (Santi *et al.*, 2008).

1.8.2.2. Pros and cons of transient nuclear transformation

- This method is incredibly quick, providing results a week after the host cell becomes infected with the bacteria ((Fischer *et al.*, 1999)

- In plant genomes, transient transformation can express micro-RNAs (miRNA) and small interfering RNAs (siRNA), which operate to interfere with the function of certain genes. Thus, it is possible to halt the activity of undesirable or superfluous genes through temporary transformation (Hwang *et al.*, 2017).

- Compared to stable transformation, transient transformation is more efficient and doesn't require time-consuming tissue culture methods (Kaur *et al.*, 2021).

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One drawback of this method is that it cannot produce the large amounts of product that are needed (Horn *et al.,* 2004).

1.9. *Solanum lycopersicum* L.

The *Solanaceae* family includes the tomato (*Solanum lycopersicum* L), which is a highly important vegetable crop worldwide (Krishna *et al.,* 2022). Tomatoes are considered berries from a botanical standpoint, yet they are considered vegetables from a nutritional one (Gautam *et al.,* 2015). With 24 chromosomes (2n), it is diploid (Causse *et al.,* 2020). Around the world, tomatoes are grown in a wide variety of colors and shapes. In the last forty years, the world's tomato-growing area has expanded by 164%, while tomato consumption has surged by 314% (Nicola, 2009).

According to the most recent FAO data, the largest tomato-growing countries are the United States, India, and China (Toni *et al.,* 2021). There are many other names for tomatoes in the world. Most people called it the "love apple" (Jenkins, 1948). In German, it is termed tomate; in Spanish, jitomate; in English, garden tomato or just tomato; and in Urdu, tamater (Waheed *et al.,* 2020). The tomato is an adaptable fruit or vegetable. It serves a variety of culinary functions. Ripe fruit can be eaten raw or processed to make a variety of goods, such as canned fruits, ketchup, soup, paste, sauce, and powder.

Consuming tomatoes improves human health (Giovannucci *et al.,* 1995). It is well-known for having biological properties that are chemo-preventive, anti-inflammatory, anti-proliferative, and anti-genotoxic (Rafi *et al.,* 2007; Scolastici *et al.,* 2007; Polivkova *et al.,* 2010). Tomatoes are low in calories and high in vitamins A and C as well as amino acids (Ray *et al.,* 2016). Lycopene, β-carotene, kaempferol, quercetin, tocopherol, luteolin, and apigenin are among the biologically active compounds found in tomatoes (Alam *et al.,* 2019; Toni *et al.*, 2021).

1.9.1. Taxonomic classification

The *Solanaceae* family, sometimes referred to as the "deadly" Nightshade family, includes tomatoes. Because of this, numerous myths about tomatoes being poisonous have been disseminated in the past. This false reputation was later disproved (Li *et al.,* 2012). Tomato was added to the *Solanum* genus by Linnaeus in 1753 under the name *Solanum lycopersicum* L.

More than 3000 species make up this family, and many of them are significant economic crops, such as tobacco, potatoes, peppers, eggplants, and petunias (Bai & Lindhout, 2007). In 1753, Carolus Linnaeus gave tomato the scientific name *Solanum lycopersicum* L. (lyco is Latin for "wolf," while persicum is Spanish for "peach"). On the other hand, Philip Miller separated the tomato and a few other species in 1754 and named the tomato *Lycopersicon esculentum* Mill (esculentum = "edible"). The complete taxonomic classification of *Solanum lycopersicum* L. is given in Table 1.1.

Table 1.1: Taxonomic classification of *Solanum lycopersicum* L. (Gautam, 2013)

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

1.9.2. Morphology

The tomato is a perennial plant with fleshy red fruits, compound leaves, and a sympodial branch (Kimura and Sinha, 2008). It has a limited lifespan (Bauer *et al.,* 2009) and a maximum height growth of only 1 to 3 m (Shukla *et al.,* 2013). The woody, glandular, hairy, coarse, and delicate stem of the tomato plant has thick, rounded leaflets and little compound leaves. All the leaves are 10–30 cm wide and 15–50 cm long, arranged in a spiral pattern. Fruits are generated by inflorescences that yield 1.5 to 2 cm long golden trumpet-shaped blooms. Fruits have colors and a diameter of 1 to 2 centimeters (Waheed *et al.,* 2020).

Warm temperatures are necessary for tomatoes to grow properly. 20°C was shown to be the ideal temperature for seed germination, whereas 25°C to 35°C is the ideal range for fruit growth. Fruit development is impacted, and its quality is diminished at temperatures below 20°C.

The tomato fruit comes in a wide range of sizes and shapes, from small and spherical to large and possibly shaped in different ways. The fruits' colors also differ. They come in the following colors: red, purple, green, or yellow (Atherton & Rudich, 2012). Figure 1.10 shows all morphological features of the tomato plant i.e., seeds, flowers, fruits and a completely grown tomato plant.
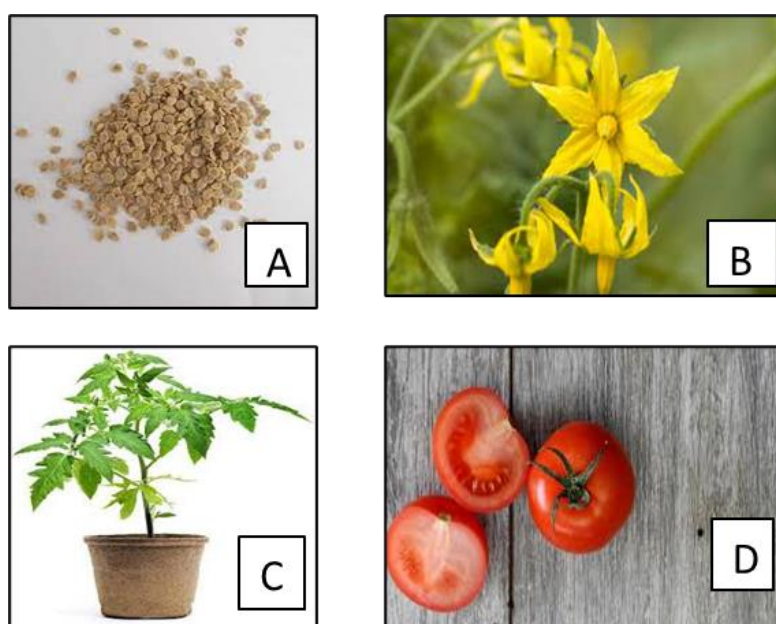
Figure 1.10: Morphology of tomato plant. (A) Seeds (B) Flowers (D) Tomato Plant (E) Tomato Fruit

1.9.3. Use in biotechnology

With one of the best-defined plant systems, the tomato is one of the most studied crops at the genetic and genomic levels. It is the most widely eaten fruit all over the world. Its consumption is being increasing day by day and its cultivation is also being increasing. It is the richest source of nutrients and provides protection against a number of health issues. Because tomato is an edible fruit so that is why it is effective to be used as an oral vaccine and it therefore helpful in boosting our immune system (Youm *et al.,* 2008). Its small genome (950 Mb), ease of vegetative and seed propagation, and cross-compatibility with numerous wild species are some of its traits

that lend itself to various genetic analyses (Di Matteo *et al.,* 2011). The tomato's broad range of mutations, homozygosity, absence of gene duplication, and capacity for asexual reproduction make it an excellent choice for use as a model plant (Alsamir *et al.,* 2021; Gerszberg *et al.,* 2015). It can be utilized as a substitute for *Arabidopsis thaliana*, which is frequently employed as a model plant because of the variety of germplasm resources and unique traits of the plant, such as the ability to produce seeds, flower, produce compound leaves, produce fleshy fruits, and have mycorrhizal roots (Carvalho *et al.,* 2011; Gerszberg *et al.,* 2015). The development of a simple yet efficient regeneration system is necessary because traditional crop improvement techniques have proven to be quite problematic. Numerous biotechnological applications, including the clonal propagation of commercial cultivars, genetic transformation, and the production of virus-free plants, have made use of tomato cultures grown in vitro (Li *et al.,* 2011; Yarra *et al.,* 2012; Namitha and Negi, 2013). Tomatoes can be used in tissue culture method to stably transform the gene of interest. The first tomato transformation caused by *Agrobacterium* was documented in 1986. Since then, several explants, including leaves, fruits, cotyledons, and hypocotyls, have been used in multiple transformation strategies for different tomato cultivars (McCormick *et al.,* 1986; Wu *et al.,* 2011). FDA approved the Flavr-Savr tomato (also known as CGN-89564), the first genetically modified food to be licensed for human consumption, in 1994. Unfortunately, the product was taken off the market in 1997 due to its delicate and bland taste (Gerszberg *et al.,* 2015). Vaccines against diphtheria, pertussis, tetanus etc. have been developed by means of genetic modification of tomato (Soria-Guerra *et al.,* 2007).

1.10. Aims and objectives

The aims and objectives of this study were as follows:

- To optimize the protocol for carrying out *Agrobacterium*-mediated stable nuclear transformation of *Solanum lycopersicum* L. nodal explants with HPV-18 *L1* gene

- To verify the transformation through several molecular biology techniques like PCR and qRT-PCR

- To check the expression of HPV-18 *L1* protein by Dot blot, Western blotting, and ELISA

2. Materials and Methods

This research study aimed to develop an efficient transformation protocol for *Agrobacterium*-mediated transformation of *Solanum lycopersicum* L cv. Rio Grande with plasmid-containing HPV-18 *L1* antigen. Furthermore, to confirm the incorporation and expression of the HPV-18 *L1* antigen in transformed plants via polymerase chain reaction (PCR) and other molecular biology techniques. This section will include information on the apparatus used as well as the methods used in this study. The current study was conducted in Plant Biotechnology Lab, Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. 4

2.1. Materials

Various apparatus (plastic and glass wares) and chemicals acquired from various manufacturers and suppliers were employed in this study. All these materials are included in table 2.1-2.6.

Table 2.1: Chemicals and reagents

Chemicals	Catalog No.
Agar (Bacteriological grade)	Bioworld, Dublin, Germany
Agar Plant TC	Phytotech lab, US
Ammonium persulphate (APS)	AnalaR^{TM}, England
Ammonium sulfate($(NH_4)_2SO_4$)	GPR, BDH laboratory supplies, UK
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$_3$) Analytical grade	Sigma-Aldrich, USA
CTAB	Oxford lab chem, India
Dimethyl sulfoxide (DMSO)	Biochem, USA
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 (87%)	AnalaR^{TM}, England
Glycine	Merck, Germany
HEPES, Free Acid (C$_8$H$_{18}$N$_2$O$_4$S)	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$_2$O)	AnalaR^{TM}, England
Methanol	Sigma-Aldrich, USA
Phenol	Merck, Germany
Potassium Chloride (KCl)	AnalaR^{TM}, England

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Propanol-1 (Propa-1-ol)	AnalaR^{TM}, England
Propanol-2 (Propa-2-ol)	AnalaR^{TM}, England
SDS (Sodium dodecyl Sulfate)	Phytotechnology Laboratories, USA
Sodium acetate (CH$_3$COONa)	AnalaR^{TM}, England
Sodium Chloride (NaCl)	AppliChem, USA
Sodium deoxycholate	Sigma-Aldrich, 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

Numerous ready to use kits and Reagents were used in our study and all of them are listed below.

Table 2.2: Ready to use kits and Reagents**.**

Reagents	Company
1kb DNA Ladder	Thermofisher, USA
dNTP set	Thermofisher, USA
3,3,5,5-tetramethylbenzidine (TMB)	Thermofisher, USA

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Chemiluminescence system	ECL® PLUS; GE Healthcare
Horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG	Abcam, USA
Protein Ladder	Thermofisher, USA
Nitrocellulose membrane	Thermofisher, USA
RNase A	Bioworld
SYBR green	Thermofisher, USA

Both nuclear and internal, forward, and reverse primers' sequences (Table 2.3) were used. Nuclear primers of HPV-18 give fragments of 1984bp length while internal primers of HPV-18 give fragments of 372bp .

Table 2.3: Primers

Primers	Sequences
HPV-18 internal forward	**5'**ATGAATAAAGTAAAATTTTATGTTTTATT**3'**
HPV-18 internal reverse	**5'**GTTTTCCATACTGATTGC**3'**
HPV-18 nuclear forward	**5'**GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGAATAAAGTAAAATTTTATGTTTTATTTA**3'**
HPV-18 nuclear reverse	**5'**GGGGACCACTTTGTACAAGAAAGCTGGGTTTA**3'**

Table 2.4: Enzymes

Enzymes	Company
Taq DNA polymerase (5U/µl)	Thermofisher, USA

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Table 2.5: Laboratory instruments

Appliances	Company
Autoclave	Uamato, USA
Balance	Ohaus Corp, USA
Blotting apparatus	Major Science, USA
Centrifuge	Eppendorf, Germany
Conventional PCR	Applied biosystems, USA
Colibri microvolume spectrometer	Titertek Berthold, Germany
Electroporator	Bio-Rad, USA
Freezer	Dawlance, Pakistan
Freezer (-70°C)	VWR, USA
Gel electrophoresis apparatus	Cleaver Scientific, USA
Gel casting device	Cleaver Scientific, USA
Gel documentation system	Alpha View SA Version 3.4.0.0, USA
Hot plate stirrer	IKA Labor Technik, Germany
Micropipettes	Eppendorf, Germany
Magnetic stirrer	VWR, USA
Minitron Incubator	VWR, USA
Orbital shaker	VWR, USA
Power supply	Biometra, USA

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Real-Time PCR	Mygo Pro, Ireland
Spectrophotometer	ThermoScientific multiskan GO
Vortex	Scientific Industries, USA
Water bath	Precision, 180 Series, USA

Table 2.6: Consumables

Consumables	Company
Glassware	Pyrex, USA
15/50 ml Falcon tubes	Corning® USA
1.5/2 ml Eppendorf tubes	Axygen Scientific, USA
Petri plates	Pyrex, USA
PCR tubes	Thermo Scientific USA
Gloves	Qualtex Malaysia
Whatman filter paper	GE Healthcare UK
Pipette Tips	Extra Gene, USA
Syringe filters (0.2 µm)	Sartorius, Germany
Microtiter plates	Costar, USA
Nitrile gloves	Gen-X, Malaysia
Surgical blades	XINDA, China
Parafilm PM-96	Bemis, USA

2.2. Methods

2.2.1. Working Precautions

Sterilized conditions are necessary for delicate activities such as plant tissue culture and transformation. Sterile conditions should be maintained throughout the process to reduce the possibility of contamination, cross-contamination, and for human safety. The following actions were done to keep such conditions in place:

- While conducting laboratory work, a lab coat was worn.

- A pair of gloves was used during solutions preparations, gel formation, working with bacterial cultures and plant materials.

- All the equipment and media, such as: MS media, LB media, distilled water, glass wares, forceps, scalpels, and blades used were sterilized by autoclaving for 20 minutes, at 121°C and 15 psi.

- Surface sterilization of the workstations was accomplished by swabbing with 70% ethanol or 95% methanol.

- Laminar Flow Hood (LFH) was sterilized by swabbing with 70% ethanol and then exposing it to Ultraviolet (UV) radiation for 20-30 minutes. All the equipment to be used (except chemicals, hormones, antibiotics, plants, seeds, and bacterial cultures) were also surface sterilized and exposed to UV radiation within LFH.

- Hands were also sprayed with 70% ethanol before working within the sterile LFH.

- After working, hands were disinfected by washing with soap or by using sanitizer.

- Contaminants were autoclaved and treated with concentrated bleach before disposal.

2.2.2. Sterilization of Laminar Flow Hood

The LFH, which has a High Efficiency Particulate Absorption (HEPA) filter, kept the working environment sterile. Before working, the LFH was thoroughly swabbed with 70% ethanol (alternative: spirit). UV was switched on for 20-30 minutes to ensure total surface sterilization.

Before getting the apparatus required for your work within the hood in the LFH, it was sprayed with 70% ethanol (alternative: spirit) and exposed to UV radiation for

20-30 minutes. After 30 minutes, the UV was turned off, and the hands were sprayed with 70% ethanol (alternative: spirit) before accessing the sterile environment of the LFH. Instruments like the forceps and scalpels were sanitized before use by soaking them in 70% ethanol and flame-drying them until they were red-hot.

2.2.3. Culture media

Different media were employed for various reasons. For seed germination, Murashige and Skoog (MS) media was employed, and different strengths of MS media with best optimized plant growth regulators (PGRs) were used for plant regeneration. For bacterial inoculations, Luria Broth (LB) medium was used, and Luria Agar (LA) media was used for streaking the bacterial inoculum. Concentrations of all medias been used are given below.

2.2.3.1. Murashige and Skoog (MS) media

Different strengths of MS media were used for germination of tomato seeds along with regeneration of explants. MS media with basal salts was utilized for tomato seed germination, while MS with vitamins was used for tomato nodes regeneration. MS was prepared by weighing specified amounts of MS with basal salts and sucrose using a weighing balance and completely dissolving them in specific amount of distilled water in a reagent bottle. A pH meter was used to measure the pH of the solution, which was maintained between 5.75 and 5.85. 0.1 N HCl or NaOH solutions were used, depending on the requirement of the media, to adjust pH. In the end, a specific quantity of Phyto-agar was measured and added to the reagent bottle. After securing the cap, the bottle was autoclaved for 20 minutes at 121°C and 15 psi. Under sterile LFH conditions, the media was put into many jars (each holding 50ml). The jars were sealed after solidification and kept at room temperature in the growth room.

Table 2.7: Composition of plant culture media

<div align="center">

½ MS Media

</div>

Chemicals	Concentration g/l
MS Media with basal salts	2.2g
Sucrose	30g

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| Agar | 8g or 0.8% |

Liquid ½ MS Media

Chemicals	Concentration g/l
MS Media with basal salts	2.2g
Sucrose	30g

Full MS Media

Chemicals	Concentration g/l
MS Media with vitamins	4.4 g
Sucrose	30g
Agar	8g or 0.8%

2.2.3.2. Bacterial culture media

Luria Broth (LB) media was used for bacterial inoculations and Luria Agar (LA) media was used for streaking the bacterial inoculum.

2.2.3.2.1. Luria Broth (LB) media

In LB media, bacteria were inoculated. A calculated amount of LB (table 2.8) was dissolved in a particular amount of distilled water in a pre-autoclaved reagent bottle. The media were autoclaved under standard conditions and kept at room temperature (25°C).

2.2.3.2.2. Luria Agar (LA) media

For the growth of bacteria (streaking) in petri plates, Luria Agar media was utilized. This media was prepared by weighing LB and bacterial agar and dissolving it in the required amount of distilled water in a reagent bottle. The prepared media was autoclaved for 20 minutes at 121°C and 15 psi.

The media was then cooled and placed in a laminar flow hood under ultraviolet (UV) radiation for ten minutes. After this the media was poured in petri plate (30ml) along with the required amounts of antibiotics. For the present study, 50mg/l kanamycin and

50mg/l rifampicin were added from the stock solutions. When the media in the petri plate had solidified, it was carefully wrapped by parafilm and kept in a growth room at 25°C.

Table 2.8: Composition of bacterial culture media

LB and LA media were used in this study. LB media was used to inoculate bacteria. Streaking of bacterial inoculum was done in LA media. The composition of both LB and LA are given below.

LB Media

Components	Concentrations
Tryptone	10g
Yeast Extract	5g
Sodium Chloride	10g

LA Media

Components	Concentrations
Tryptone	10g
Yeast Extract	5g
Sodium Chloride	10g
Bacteriological grade agar	1%

2.2.4. Preparation of Stock solutions

In this research, stocks of different plant hormones and antibiotics were used in different concentrations. Their composition is provided in table 2.9.

Table 2.9: Stock solution concentration of different hormones and antibiotics

Hormone specific for our plant is zeatin and its stock concentrations used in this study is given below.

Hormones

Stock solutions	Concentrations
Zeatin	2mg/ml

Different antibiotics used in this study and their stock concentrations are given below.

Antibiotics

Stock solutions	Concentrations
Rifamycin	20mg/ml
Kanamycin	50mg/ml
Acetosyringone	100Mm
Cefotaxime	250mg/ml

2.2.4.1. Preparation of stock solution of hormones

2.2.4.1.1. Zeatin

To prepare 2mg/ml stock of zeatin, 200mg powdered zeatin was weighed, and it was added in 50ml falcon tube in laminar flow hood. To dissolve this, 10ml sterile distilled water was added to it. This stock solution was filter sterilize using a syringe filter with a diameter of 0.2μm. And was stored in small aliquots at -20°C.

2.2.4.2. Preparation of stock solution of antibiotics

2.2.4.2.1. Kanamycin

To prepare 50mg/ml stock solution of kanamycin, 500mg kanamycin powder was weighed and added to 50ml falcon tube in laminar flow hood. Then 2ml of autoclaved distilled water was added into the falcon tube. This is then shaken for several minutes. Upon complete dissolution, the volume was raised to 10ml with autoclaved distilled water. This stock solution was filter sterilized using a syringe filter that was 0.2μm in diameter and was stored in small aliquots at -20°C.

2.2.4.2.2. Rifamycin

To prepare 20mg/ml stock solution of rifamycin, 200mg rifamycin powder was weighed and dissolved in 10ml dimethyl sulfoxide (DMSO) in 15ml falcon tube in

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laminar flow hood. As rifamycin is not soluble in distilled water, its dissolved in DMSO. It was then shaken for several minutes. Upon complete dissolution, this stock solution was filter sterilized using a syringe filter that was 0.2µm in diameter and was stored in small aliquots at -20°C.

2.2.4.2.3. Acetosyringone

To prepare 100mM stock solution of acetosyringone, 196.2mg acetosyringone powder was dissolved in 10ml DMSO in 15 ml falcon tube. It was then shaken for several minutes. Upon complete dissolution, this stock solution was filter sterilized using a syringe filter that was 0.2µm in diameter and was stored in small aliquots at -20°C.

2.2.4.2.4. Cefotaxime

To prepare 250mg/ml stock solution of cefotaxime, 1g cefotaxime powder was dissolved in 4ml distilled water in laminar flow hood. It was then shaken for several minutes. Upon complete dissolution, this stock solution was filter sterilized using a syringe filter that was 0.2µm in diameter and was stored in small aliquots at -20°C.

2.2.5. Germination of seed

2.2.5.1. Plant material

Tomato seeds cv. Rio Grande were used in this research work which were purchased from Awan Seed Store, Rawalpindi, Pakistan. They were stored in sealed falcon tubes at room temperature (25°C) in a cool and dry environment.

2.2.5.2. Sterilization of tomato seeds

Tomato seeds were surface sterilized within the sterile conditions of LFH before inoculating them on the germination media. This step was done to minimize the possibility of bacterial and fungal contamination which would otherwise reduce the seed germination ability. Sterilization details of tomato seeds are given below:

Table 2.10: Seed sterilization method used

Seeds sterilization method	Chemicals used	Time of treatment
SM 1	0.1% mercuric chloride	15-20 seconds
	Autoclaved distilled water	3 minutes each

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2.2.5.3. Germination of tomato seeds

Tomato seeds were germinated using ½ MS media which was prepared and autoclaved under standard conditions and poured into jars which were then sealed and kept in growth room at 25ºC one day before germinating seeds on that media. Before germinating seeds all the apparatus was kept in UV for 20 minutes except seeds. After that seeds were inoculated on media in jars and the jars were sealed with parafilm and stored in growth room at 25ºC.

2.2.5.4. Preparation of explants

Tissue culture was used for regenerating the plant using an explant, a small fragment of node and leaf. Nodal explants were tissue cultured for regeneration and transformation in this research.

2.2.5.5. Preparation of nodal explants

Plants that were cultivated in vitro for 2-3 weeks were employed to prepare nodal explants. Leaves were cut from nodes with sterile forceps and a scalpel, and nodes were sliced into 1.5-2 cm long pieces.

2.2.5.6. Regeneration of tomato explants

Full MS media having Zeatin was used for the regeneration of tomato explants. Composition of media for regeneration of tomato explants is given in table 2.9.

Table 2.11: Composition of media for regeneration of tomato explants

S. no	Components	Composition
1.	MS strength	Full MS
2.	Hormones	Zeatin (2mg/ml)

2.2.6. Preparation of media for transformation

Three different types of media i.e. co-cultivation, washing and selection media were prepared for the stable transformation of *Solanum lycopersicum* L. The description of these media is given below.

2.2.6.1. Preparation of co-cultivation media

Under the sterile conditions of LFH, 200µM acetosyringone was added to the autoclaved tomato regeneration media in a reagent bottle to prepare the co-cultivation media. Then this media was poured into petri plates and the plates were kept for solidifying. Upon solidification, petri plates were wrapped with parafilm and were kept in growth room at 25ºC.

2.2.6.2. Preparation of washing media

As a washing medium, liquid ½ MS was used. In a reagent bottle, a calculated amount of MS with basal salts and sucrose was mixed with distilled water. The pH was maintained, and it was autoclaved under standard conditions. Washing was done three times in a falcon tube with 1000mg/l cefotaxime in 30ml liquid ½ MS medium. The final washing was done using autoclaved distilled water that did not include cefotaxime.

2.2.6.3. Preparation of selection media

To prepare selection media, 250mg/l cefotaxime and 75mg/l kanamycin were mixed into tomato regeneration media in a reagent bottle in LFH. This media was then poured into petri plates and allowed to solidify. Petri plates were wrapped with parafilm and kept at 25°C in the growth room until further usage.

2.2.7. First Transformation: genetically modified *Agrobacterium tumefaciens* strain

In this bacterial transformation, *Agrobacterium tumefaciens* were transformed with a binary vector pGWB5 which contained our integrated gene of intertest i.e., HPV-18 *L1*.

2.2.7.1. Preparation of *Agrobacterium tumefaciens* growth culture

After preparing LA plates containing 50 mg/l kanamycin and 50 mg/l rifamycin, *Agrobacterium* strain GV3101 was streaked/grown on them in LFH. They were then stored in a 25°C growth environment for 2-3 days until bacteria colonies developed. A single colony of bacteria was picked using a sterile inoculating loop and placed in a freshly produced and autoclaved liquid LB medium with 50mg/l kanamycin and 50mg/l rifamycin for selection, and the LA plates were refrigerated at 4°C to stop

further bacterial growth. This liquid LB medium containing our inoculum was stored in growth room at 25°C for 1-2 days till bacterial growth appeared. The OD600 value was then determined using a spectrophotometer (Thermo Scientific Multiskan GO).

2.2.7.2. Preparation of Electro-competent cells

We prepared electro-competent cells of GV3101 *Agrobacterium* strain in this research work. Electro-competent cells were prepared by transferring the bacterial inoculum (in LB media) into a 50ml falcon tube. Then this inoculant was centrifuged at 2400 rpm for 20 minutes at 4ºC. 40ml chilled distilled water was added to the pellet which was obtained after centrifugation to dissolve the pellet. The centrifugation step was repeated at 2400 rpm for 20 minutes at 4ºC, this time the pellet was dissolved in 20ml chilled distilled water. Again, centrifugation was done at 2400 rpm for 20 minutes at 4 ºC and the pellet was dissolved in 10ml chilled distilled water. In the end, centrifugation was done, and the pellet was dissolved in 500µl of ice-cold 10% glycerol (10ml glycerol: 90ml distilled water). After mixing gently, 60µl aliquots were created and stored at temperature of -70ºC.

2.2.7.3. Electroporation

Electro-competent cells were electroporated with a plasmid (7µl) using the electroporator (Bio-Rad, USA). The electro-competent cells were placed on ice to get thawed. 7µl plasmid was added to the 50µl of these cells and the sample was transferred to the cuvette. The PULSE button was pressed on the electroporator for the electric shock of 1.8kV. 400µl LB media (table 2.8) was added to the cuvette. The sample was then transferred to the new eppendorf tube and was placed in the shaking incubator at 37ºC for 3 hours. After 3 hours, 30µl of this sample was used and streaked on LA plates having the required antibiotics and 100µl from the same sample was used for inoculation in LB having the suitable antibiotics (table 2.12). The plates and the inoculated LB were placed overnight at 28ºC.

Table 2.12: Antibiotics for the selection of transformed *Agrobacterium tumefaciens*

Antibiotics	Stock concentration	Working concentration
Kanamycin	50mg/ml	50mg/ml
Rifamycin	50mg/ml	20mg/l

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2.2.7.4. Binary vector pGWB5 containing HPV-18 *L1*

Agrobacterium tumefaciens strain GV3101 was transformed with pGWB5 binary vector which was containing our gene of interest i.e., HPV-18 *L1*. This transformed *Agrobacterium* was used to transform *Solanum lycopersicum* L. T-DNA region of the pGWB5 binary vector consisted of our gene of interest i.e., HPV-18 *L1* with 35S promoter and NOS terminator as shown in Figure 2.1.
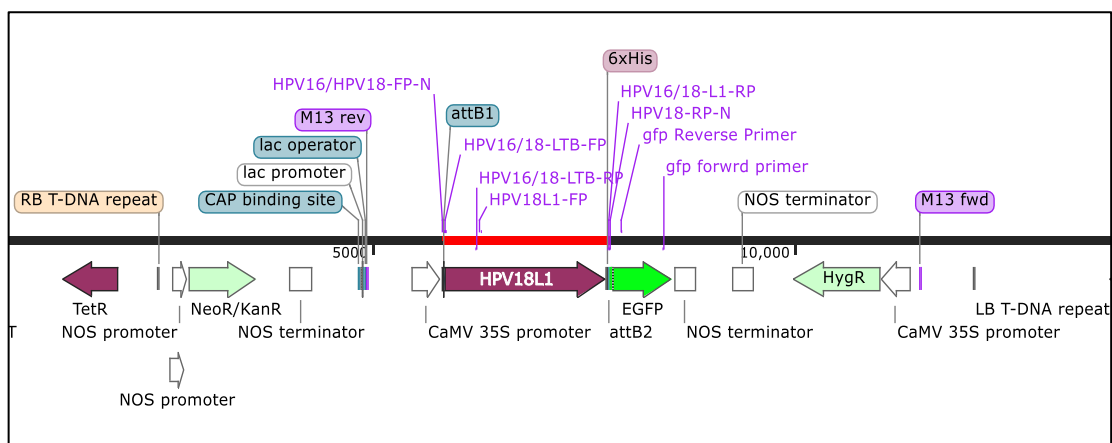
Figure 2.1: Schematic representation of final HPV-18 expression vector

2.2.8. Second transformation-transgenic *Solanum lycopersicum* L

Solanum lycopersicum L was transformed by using stable nuclear transformation method.

2.2.8.1. *Agrobacterium*-mediated stable transformation of *Solanum lycopersicum* L

2.2.8.1.1. Co-cultivation

In a flask, a GV3101 strain of *Agrobacterium tumefaciens* harboring the binary vector pGWB5 was inoculated in 50ml of freshly prepared and autoclaved LB media (table 2.8). For selection, 50mg/l kanamycin and 50 mg/l rifamycin was added to this LB media. For 48 hours, the flask was kept at 25°C in the growth room. When the OD was between 0.6 - 0.8, 1ml of this inoculum was inoculated in a flask with 50ml of freshly prepared and autoclaved LB containing 50mg/l kanamycin and 50mg/l rifamycin, as well as 200µl acetosyringone. For one day, the flask was placed in a 25 °C growth room. The OD was adjusted between 0.6 - 0.8 the following day. This inoculated LB was transferred to a 50ml falcon tube and centrifuged at 5500×g at

room temperature for 20 minutes. The supernatant was discarded, and the pellet was dissolved in 50ml of liquid ½ MS media (table 2.7) within LFH under sterile conditions. This infection media contained 200µM acetosyringone. Nodal explants were prepared and immersed for 8 minutes in this bacterial suspension. They were placed on autoclaved filter paper after infection. They were then transferred to co-cultivation media plates after drying. 10-15 nodal explants were co-cultivated in each plate. The plates were sealed with parafilm, and they were placed in the growth room at 25 ± 2 ºC in dark for different time periods i.e., 2 and 3 days. Control (no infection) was also shifted to co-cultivation media. This work was done in replicates.

2.2.8.1.2. Selection

After two days on co-cultivation media, all the nodal explants were washed three times by liquid ½ MS media containing 1000mg/l cefotaxime for five minutes each and the fourth time by autoclaved distilled water except the control where wild type nodal explants were shifted to 75mg/l kanamycin selection plate without any washing. After these washing steps of infected nodal explants, these explants were kept on autoclaved filter paper to dry. Upon drying, these explants were shifted to selection media plates containing 75mg/l kanamycin and 250mg/l cefotaxime. 6-7 infected nodal explants were shifted to each selection plate which was sealed by parafilm and kept in the growth room under light at 25ºC. After every week, these infected explants were washed three times by liquid ½ MS media containing 1000mg/l cefotaxime for five minutes separately and the fourth time by autoclaved distilled water and these were shifted to new petri plates having fresh selection media. This whole work was done in the sterile conditions of Laminar Flow Hood. The tomato plants which were transformed were regenerated on selection media. Their shoot regeneration and callus formation efficiency were calculated by using the formula described as follows:

$$\text{Shoot regeneration efficiency (\%)} = \frac{\text{Number of shoots regenerated}}{\text{Total number of explants inoculated}} \times 100$$

Their callus formation efficiency was calculated by the following formula:

$$\text{Callus forming efficiency (\%)} = \frac{\text{Number of calli formation from explant}}{\text{Total number of explants inoculated}} \times 100$$
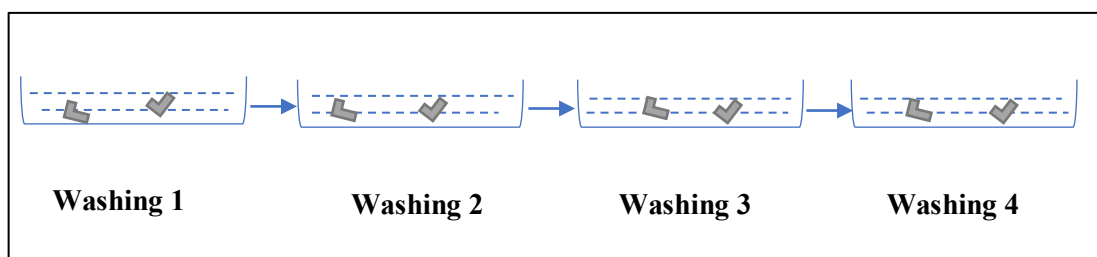
Figure 2.2: Washing steps after co-cultivation: Washing 1: Liquid ½ MS with 500mg/l cefotaxime; Washing 2: Liquid ½ MS with 250mg/l cefotaxime; Washing 3: Liquid ½ MS with 125mg/l cefotaxime; Washing 4: Autoclaved distilled water.

2.2.9. Techniques of molecular biology to verify transformation

In this present study, transformation of plants with HPV-18 *L1* were confirmed by conventional PCR analysis. Copy number of integrated HPV-18 *L1* gene was determined by performing quantitative real-time PCR (qRT-PCR) technique. Bradford assay was performed for protein estimation after protein purification from plants. In transgenic plants, expression of HPV-18 *L1* protein was confirmed by performing Dot blot assay and western blotting. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was performed to quantify the HPV-18 *L1* protein in total soluble protein, extracted from transgenic plants.

2.2.9.1. Plasmid isolation from transformed *Agrobacterium tumefaciens*

Isolation of plasmid was done by the protocol reported by (Russell and Sambrook, 2001) with several modifications. The inoculated LB media (O.D.$_{600}$, 0.6-0.8) was transferred to a fresh 50ml falcon tube. This culture was centrifuged for 10 minutes at 4ºC at 14000 rpm, 200µl solution 1 was added to dissolve the pellet (Annexures 2.1) which was then transferred to a fresh eppendorf tube. 400µl solution 2 (Annexures 2.1) was added. The tube was inverted several times and then placed on ice for 5 minutes. 300µl of lysis solution 3 (Annexures 2.1) was added to it. The components were mixed thoroughly by inverting the tube for several minutes and then it was placed on ice for 5 minutes.

After that it was centrifuged for 5 minutes at 4ºC at 14000 rpm. 600µl supernatant was collected and transferred to a fresh eppendorf tube. An equal volume of phenol-chloroform was added to the supernatant. Then the tube was centrifuged for 5 minutes at 4ºC at 14000 rpm. Two layers were formed, the upper layer was aqueous and lower

layer was organic. The upper aqueous layer was transferred to a new eppendorf tube, 600µl of isopropanol was added to the eppendorf tube containing aqueous layer, and then this eppendorf was kept on ice for one hour.

After an hour, it was centrifuged at 4 ºC for 5 minutes at 14000 rpm. The supernatant was discarded. The pellet was washed with 70% 1ml ethanol. Then it was centrifuged one more time for 5 minutes at 4 ºC at 14000 rpm. Ethanol was removed and the pellet was allowed to dry. 20µl T.E buffer (Annexure 2.1) was added along with RNase A, and it was stored at -20 ºC.

2.2.9.2. Isolation of plant genomic DNA

Genomic DNA was extracted by cetyltrimethylammonium bromide (CTAB) procedure reported by (Russell & Sambrook, 2001). DNA was isolated from both wild-type and transgenic plants. CTAB buffer (Annexure 2.2) was prepared one day before the DNA extraction from plants. The sample (nodes) was placed overnight at -20°C. Next day, it was ground into fine powder with liquid nitrogen. The powdered sample was transferred to freshly autoclaved eppendorf. The temperature of the water bath was pre-adjusted at 65°C.

Before use, CTAB was placed in water for 30 to 60 minutes. To the sample in Eppendorf, 700µl of CTAB buffer was added. The homogenate was vortexed and left in a preheated water bath for an hour. 600µl of cold phenol-chloroform-isoamyl alcohol (Annexures 2.2) was added to each sample after one hour. The components were homogenized thoroughly by inverting the Eppendorf tubes repeatedly for 30 minutes. Then centrifugation was done at 27ºC, 14000 rpm for 15 minutes.

Supernatant was collected to a new eppendorf tube, and the pellet was discarded. Cold isopropanol was added to the sample and was then incubated overnight at -20 ºC. Next day, the sample was centrifuged for five minutes at 27 ºC and 14000 rpm. Supernatant was discarded and pellet was collected. 1ml of chilled 70% ethanol (Annexure 2.2) was added and the eppendorf was centrifuged for five minutes at 27 ºC and 14000 rpm.

This washing step was repeated, and supernatant was discarded. Finally, the pellet was dry in air by inverting it for several minutes. In the end 20 µl T.E buffer (Annexure 2.1) and 1µl RNase was added. Then it was stored at -20 ºC.

2.2.9.3. Micro-volume quantification of DNA concentration

To find the concentration of DNA isolated from tomato, Colibri microvolume spectrophotometer using a highly sensitive approach called NanoDrop micro-volume quantification (Desjardins and Conklin, 2010) was used. Firstly, the bottom and top optical panels of the micro-volume spectrophotometer was cleaned by 2-3µl of deionized water onto the bottom optical panel. The upper panel got into contact with deionized water when the lever arm was fully closed. After that, the liver arm was raised and both optical panels were cleaned with dry, clean lab wipes free of fur. After opening nanodrop software, 'Nucleic Acid Application' was pressed. 1µl of T.E buffer was spread over lower optical panel to obtain a blank reading. After lowering the lower arm, the option labelled ′Blank′ was selected. After the completion of blank measurement, both optical panels were cleaned by a lab wipe. To quantify DNA samples, the right constant was selected. 1µl of DNA sample was poured onto lower optical panel. The 'measure' was selected in the software application after closing the lever arm. The concentration of DNA was automatically calculated by the software.

2.2.9.4. Polymerase Chain Reaction (PCR)

In order to verify the successful transformation and integration of HPV-18 *L1* gene into the nuclear genome of tomato, PCR was performed. 25µl volume of PCR master mix was prepared for 1X PCR. Positive control (HPV-18 *L1* containing plasmid) and wild type plant was used as negative control. The expected size of amplicon was 1984 bp by using HPV-18 *L1* nuclear primers and 372bp by using HPV-18 *L1* internal primers. The composition of master mix as well as standard conditions for PCR are given in table 2.13 and 2.14 respectively.

HPV-18 *L1* nuclear forward primer

5'GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGAATAAAGTAAAATTT TATGTTTTATTTA**'3**

HPV-18 *L1* nuclear reverse primer

5'GGGGACCACTTTGTACAAGAAAGCTGGGTTTA**3'**

HPV-18 *L1* internal forward primer

5'ATGAATAAAGTAAAATTTTATGTTTTATT**3'**

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HPV-18 *L1* internal reverse primer

5'GTTTTCCATACTGATTGC**3'**

Table 2.13: For 1X PCR, composition of master mixture

Reagents	Concentration ((µl)
PCR water	18
Taq. Buffer	2.5
MgCl$_2$	1.5
DNTPs	0.5
Forward primer	0.5
Reverse primer	0.5
Taq. Polymerase	0.5
Template	01

Table 2.14: For PCR, standard conditions

Steps	Temperature (ºC)	Time (min:sec)	
Initial denaturation	95	05:00	
Final denaturation	95	00:45	
Annealing	49	01:00	**35 cycles**
Initial extension	72	02:00	
Final extension	72	10:00	
Hold	04	∞	

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PCR profile with temperature and time at different stages of reaction is shown in table 2.14. The annealing temperature was 49°C for internal primers and 62.8°C for nuclear primers. The total PCR reaction had 35 repeated amplification cycles.

2.2.9.5. Agarose Gel Electrophoresis

Agarose gel electrophoresis was done to check the presence of different fragments of DNA samples. Agarose form pores that separate the different fragments of DNA based on their sizes such that the smaller fragments face less resistance and thus move further from the wells as compared to the larger ones. The pore size is determined by the concentration of agarose used for making the gel and the concentration of agarose depends on the length of DNA fragments to be separated. The lesser the size of the DNA fragment, higher would be the concentration of agarose used to prepare the gel and vice versa. Thus, there is an inverse relation between the two factors.

2.2.9.5.1. Preparation of Agarose gel

To prepare a 1% agarose gel 1g of agarose powder was added to 100ml of 1X TBE buffer (Annexure 2.3) in a reagent bottle. The solution was then heated for 2 minutes in an oven to dissolve agarose completely. It was brought to room temperature. Then 7µl of ethidium bromide (Annexure 2.3) was added. The gel was poured into a gel casting tray and a comb was inserted to create wells for loading sample. It was allowed to set for 15-20 minutes at room temperature. After it has solidified completely, the comb was removed, and the gel was placed in the gel tank having 1X TBE running buffer (Annexure 2.3).

2.2.9.5.2. Sample preparation

Samples were prepared before loading on the gel. This step involves mixing all the samples with the loading dye (Annexure 2.3). The loading dye consists of bromophenol blue and sucrose. Bromophenol blue helps to keep track of the DNA samples as they run towards the positive electrode on the gel whereas sucrose binds to DNA, increases its density, allowing it to settle more quickly and opposes diffusion.

2.2.9.5.3. Electrophoresis and imaging

The PCR products were confirmed through agarose gel electrophoresis. All PCR samples including the transgenic samples as well as the wild-type samples were loaded into the wells. Before loading the gel, 7µl of PCR products were mixed with

3µl of loading dye (Annexure 2.3). 1kb ladder (Cat No: SM0314, Thermo Scientific, USA) (figure 2.3) was used for checking the size of DNA sample. Each sample was carefully loaded into distinct wells. After the samples were loaded, the lid of the gel tank was closed, power supply was connected, and electrophoresis was done for 60 minutes at 110 volts and 500mA. Upon successful running, DNA bands were visualized under UV light, using gel documentation system.
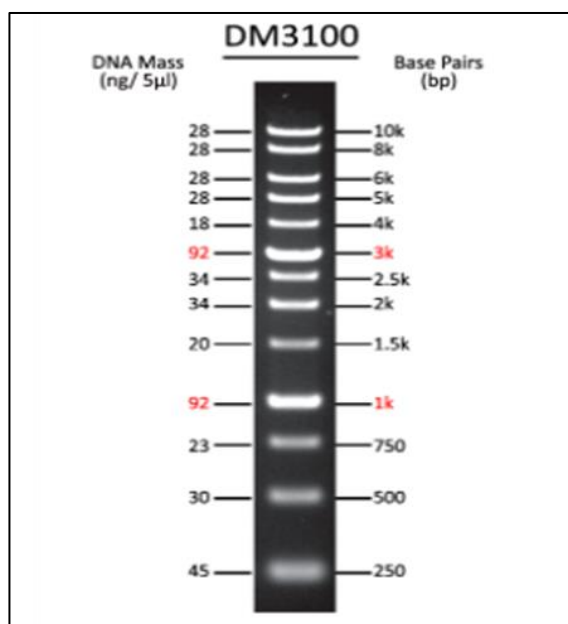
Figure 2.3: 1kb DNA ladder for Agarose Gel Electrophoresis (Taken from: www.gentechbio.com)

2.2.9.6. Quantitative Real Time (qRT) PCR

After successful transformation, the level of expression of a transgene within a plant cell was checked through qRT-PCR by following the protocol described by (Kang *et al*., 2012). qRT PCR was carried out using MyGo Pro Real time PCR (Stokesley Middlesbrough, UK). DNA samples of both wild-type and transgenic were used. 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 2.13. Unlike conventional PCR, the total reaction mixture was 10µl. 9.5µl of this reaction mixture was added to sterile PCR tubes. To this, 0.5µl of DNA sample was added. All the PCR tubes were labelled before placing them in the instrument. The primers used are provided as follows.

HPV-18 *L1* internal forward primer

5'ATGAATAAAGTAAAATTTTATGTTTTATT**3'**

HPV-18 *L1* internal reverse primer

5'GTTTTCCATACTGATTGC**3'**

Table 2.15: For qRT-PCR, composition of reaction mixture

Components	Concentration for 1X (µl)
Forward primer	0.25
Reverse primer	0.25
DNA	0.5
SYBR Green	5
PCR water	4

The conditions for qRT-PCR reaction with program names are mentioned in table 2.16.

Table 2.16: Condition for qRT-PCR

The following conditions were maintained for qRT-PCR. Initially there was a hold at 95°C. Three steps of amplification were done i.e. denaturation, annealing and extension. Annealing temperature is specific to HPV-18 internal primers i.e. 49°C. after extension pre-melt hold was done at 95°C. high resolution melting has initial and final stage. At Initial stage, temperature was maintained at 59°C and at final stage temperature was maintained at 95°C. All these conditions were set at MyGo Pro Real time PCR program and at the end of the running, it gave us the copy number of transgene and we confirmed the successful transformation.

Programs name	Temperature (°C)	Ramp (°C/s)	Hold (s)
Hold	95	4	600

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3 steps amplifications	**Denaturation**	95	5	10
	Annealing	49	4	45
	Extension	72	5	15
Pre-melt hold		95	51	15
High resolution melting	**Initial stage**	59	4	60
	Final stage	95	0.05	15

2.2.9.7. Protein extraction

To extract the total soluble protein (TSP), plant tissue (nodes) of both transformed and wild type tomato plants were cut into 100mg small pieces. These pieces were ground into fine powder in liquid nitrogen using mortar and pestle. To this powder, 500µl of protein extraction buffer (Annexures 2.4) was added. After mixing properly, the mixture was transferred to freshly autoclaved Eppendorf tube. It was vortexed and centrifuged at 20,000 g for 10 minutes at 4 ℃. The supernatant was collected in the new Eppendorf tube and the pellet was discarded. The total soluble protein (TSP) fraction of this collected supernatant was centrifuged again at 20,000 g for 10 minutes at 4 ℃ to remove cellular debris. The supernatant was transferred to the new Eppendorf tube and stored at -20 ℃.

2.2.9.8. Bradford assay (Protein quantification)

TSP concentration was determined using Bradford's assay after protein extraction. The standard curve was plotted using Bovine Serum Albumin (BSA) as a standard. A 2mg/ml BSA stock solution was prepared, which was then used to make five different BSA dilutions (Annexure 2.5). As a control, protein extraction buffer (Annexure 2.4) was used. A 96 well plate was loaded with 20µl of blank, BSA dilutions, and samples. Following that, 200µl of Bradford's reagent was added to each well containing samples. The plate was wrapped in aluminum foil and left in the dark for 30-60 minutes. After incubation, absorbance at 595nm was measured with a

spectrophotometer. Figure 2.4 shows a standard curve plotted using the absorbance values of BSA dilutions. The concentrations of each protein sample were determined using the TREND formula on an excel sheet based on this standard curve.
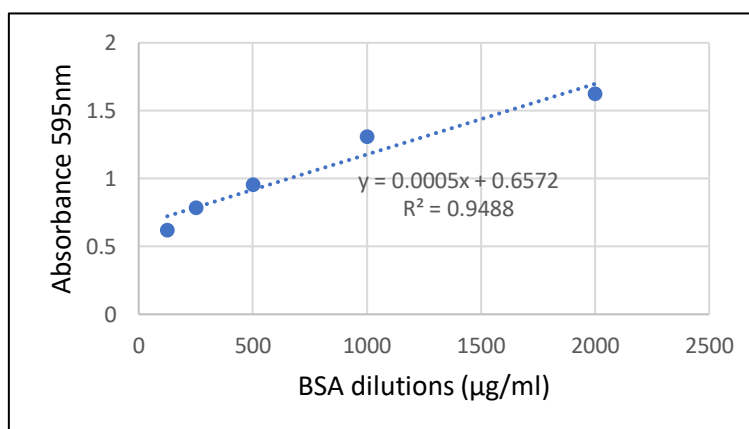
Figure 2.4: Standard curve of BSA dilutions for protein estimation.

2.2.9.9. Dot blot assay

A dot blot assay is simple and rapid. It works on the same principle as many other immunological techniques: a specific antigen is identified and bound by a specific antibody. A dot blot assay was used to confirm the expression of the HPV-18 *L1* antigen in transgenic plants. Blotting, blocking, primary antibody treatment, secondary antibody treatment, and visualization were all carried out according to the standard protocol specified by the Abcam USA protocol.

2.2.9.9.1. Blotting

A nitrocellulose membrane was employed for dot blotting. Using forceps to remove the protective covering, 2µl 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. Lead pencil was used to mark the blotted areas. Then blocking was done as described below to block non-specific sites.

2.2.9.9.2. Blocking

20 ml of blocking buffer (Annexure 2.6) was put onto the membrane and incubated at room temperature, for 30 to 60 minutes with gentle shaking to block the non-specific sites. The blocking buffer was discarded following incubation. The membrane was washed with TBST buffer to prevent excessive BSA binding (Annexure 2.7).

2.2.9.9.3. Primary antibody treatment

The primary antibody used was MD2H11 monoclonal antibody specific to *L1*. Primary antibody was prepared from stock in TBST buffer (Annexure 2.7) at a working dilution of 1:10,000 (Annexure 2.8). Membrane was kept in primary antibody for 30 minutes at room temperature. After treatment, washing was done thrice with TBST.

2.2.9.9.4. Secondary antibody treatment

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam, USA) was used as the secondary antibody. The membrane was incubated with a secondary antibody for 30 minutes with gentle shaking at room temperature. After incubation, the membrane was washed thrice with TBST buffer.

2.2.9.9.5. Visualization

The horseradish peroxidase (HRP) enzyme's chemiluminescent substrate (Cat No. WBKLS0500, Merck Millipore, Germany) was used for visualization. Luminol and hydrogen peroxidase were combined in a 1:1 ratio. For 1 minute at room temperature, the membrane was allowed to incubate with the substrate in the dark. After incubation, a chemiluminescence system (ECL®PLUS; GE Healthcare) was used to visualize the membrane.

2.2.9.10. Western blotting

The purified proteins were separated according to size using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isolated proteins were then transferred onto nitrocellulose membrane. After successful transferring, the nitrocellulose membrane was incubated with a primary antibody and a secondary antibody under ideal conditions to detect the target protein's presence. Protein samples from transgenic tomato plant tissues were used for the western blotting technique. Wild-type plants were used as negative controls.

2.2.9.10.1. SDS-PAGE

The apparatus was thoroughly cleaned and assembled. There were two gels prepared: 10% resolving gel and a 5% stacking gel. First, 10% Resolving gel (Annexure 2.10) was made and poured between the two glass plates. To linearize the gel surface, 1ml

isopropanol was placed above the resolving gel. The gel was allowed to completely solidify. Following that, 5% stacking gel (Annexure 2.11) was made. Before pouring the stacking gel over the resolving gel, the isopropanol was fully removed. The gel was allowed to set before the comb was inserted.

2.2.9.10.2. Preparation and loading of protein samples

For the preparation of protein samples, we used the 4X sample buffer (Annexure 2.12). 20µl samples were mixed with 4µl sample buffer in a sterile Eppendorf tube to make 24 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 2.13). 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.

2.2.9.10.3. Electrophoresis

After loading the sample, the gel tank's lid was shut, and the positive and negative electrodes were wired up to the power supply. The gel was first run for 20 minutes at 80 volts, and after that, for another 90 minutes or until the gel was completely run, the voltage was raised to 110 volts. The gel was next subjected to western blotting after it had run.

2.2.9.10.4. Transfer of protein

The semi-dry method was used for the protein transfer. For this, a semi-dry blotting apparatus was employed. Twelve Whatman's filter papers, cut to the size of the separating gel, were needed to transfer one gel. The transfer buffer (Annexure 2.14) was applied to these filter papers and the nitrocellulose membrane.

As shown in figure 2.5 the sandwich was made by lining up six filter papers, the nitrocellulose membrane, the separating gel and the remaining six filter papers from the positive terminal (anode) to negative terminal (cathode). Remove air bubbles using a roller because they would reduce the effectiveness of the transfer. The power source was attached to the transfer device's terminals, and the voltage was set to 12 volts for 60 minutes.
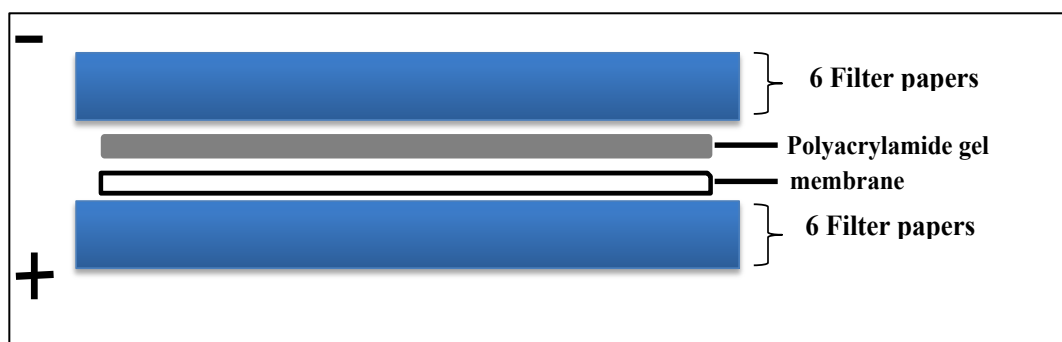
Figure 2.5: Transfer assembly for transfer of one gel

2.2.9.10.5. Blocking

Blocking is used to prevent non-specific primary antibody binding. For this, blocking solution (Annexure 2.6) is used. At room temperature, the membrane is immersed in the blocking solution for 60 minutes while being continuously shaken. After an hour, the blocking solution is removed, and the membrane is continuously stirred while being washed three times with TBST wash buffer (Annexure 2.7).

2.2.9.10.6. Treatment with primary antibody

The primary antibody used was MD2H11 monoclonal antibody specific to *L1*. A working dilution of 1:10,000 (Annexure 2.8) was prepared in TBST from stock. At 4°C, the membrane was incubated with the primary antibody overnight. It was washed three times with a wash buffer after the treatment (Annexure 2.7).

2.2.9.10.7. Treatment with secondary antibody

House-radish peroxidase (HRP) conjugated goat anti-mouse IgG was used as the secondary antibody. The membrane was incubated with the secondary antibody (Annexure 2.9) for one to two hours at room temperature while being constantly shaken. After secondary antibody treatment, washing was done with wash buffer (Annexure 2.7)

2.2.9.10.8. Visualization

Chemiluminescent substrate (Cat No. WBKLS0500, Merck Millipore, Germany) was used for visualization. Equal volumes of Luminol and hydrogen peroxide were combined. The substrate was applied to the membrane for two minutes while it was

dark. Using the gel documentation system (Alpha View SA Version 3.4.0.0), the blot was observed after incubation.

2.2.9.11. ELISA

To determine the protein concentration in the samples, indirect ELISA was performed. Indirect ELISA was carried out using a 96-well plate. ELISA extraction buffer (Annexure 2.15) was used to prepare the samples. The samples were placed into individual wells and the plate was incubated at 4°C for overnight. It was cleaned with a wash buffer (Annexure 2.7) three times after that. 200µl of blocking solution (Annexure 2.6) was added to each well. The plate was incubated for two hours at room temperature. The wells were cleaned three times with wash buffer (Annexure 2.7) after the blocking solution was removed. The plate was coated with 100µl of primary antibody (Annexure 2.8), and then incubated at room temperature for two hours. After each well had been washed three times with wash buffer (Annexure 2.7), 100µl secondary antibody (Annexure 2.9) was added. After an hour of incubation at room temperature, the plate was rinsed with wash buffer (Annexure 2.7). to get rid of any unbound antibody. Enzyme-specific substrate, called TMB (Cat No. A3840, AppliChem, Germany), was added to each well and the plate was incubated again at room temperature. After 20-30 minutes, as the blue color developed 0.1 N H_2SO_4 (stop solution) was added, and absorbance was measured at 595 nm.

Annexures

Annexure 2.1: Solutions composition for plasmid isolation

Solutions	Components	Concentration
Solution I	Glucose	50mM
	Tris	25mM, pH 08
	EDTA	10mM, pH 08
Solution II	SDS	01%
	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)	01M
	EDTA	0.5M

Annexure 2.2: Solutions for DNA isolation 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	01ml
70% Ethanol	Ethanol	70ml
	Water	30ml
T.E buffer	Tris HCl (pH 8)	01M
	EDTA	0.5M

Annexure 2.3: Solution for Agarose Gel Electrophoresis

TBE buffer recipe 10X (pH 8.0)

Components	Concentration (g/L)
Tris base	110
Boric Acid	55
EDTA	9.3

1X TBE buffer

Components	Concentration
10X TBE buffer	10ml
Distilled water	90ml

Ethidium Bromide (10mg/ml)

Components	Concentration
Ethidium Bromide	1g
Distilled water	100ml

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Loading dye

Components	Concentration
Bromophenol blue	0.25g
Distilled water	100ml

Annexure 2.4: Protein extraction buffer

Components	Concentrations
Sodium chloride	300mM
Ascorbic acid	20mM
Sodium metabisulphite	10mM
Tris (pH 08)	100mM

Annexure 2.5: BSA dilutions for Bradford assay

Concentration (µg/µl)	BSA (µg)	Distilled water (µl)
0	0	250
125	31.25	250
250	62.5	250
500	125	250
1000	250	250
2000	500	0

Annexure 2.6: Blocking buffer

Components	Concentration
BSA	5g
TBST buffer	100ml

Annexure 2.7: TBST buffer

Components	Concentration
Tris HCl	3.08g (pH 7.6)
NaCl	8.775g
Tween-20	1ml
Distilled water	1L

Annexure 2.8: Dilution of primary antibody

Components	Concentration in 30ml
MD2H11	3µl
Sodium azide	0.015g
BSA	1.5g
TBST	30ml

Annexure 2.9: Dilution of secondary antibody

Components	Concentration in 30ml
HRP-conjugated goat anti-mouse IgG	3µl
BSA	1.5g
TBST	30ml

Annexure 2.10: Resolving gel (10%)

Components	Concentration
Distilled water	4.17ml
30% stock acrylamide solution	3.35ml
4X Resolving tris solution	2.5ml

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10% ammonium persulphate	100µl
TEMED	20µl

Annexure 2.11: Stacking gel (5%)

Components	Concentration
Distilled water	6.76ml
30% Stock acrylamide solution	2ml
4X Stacking tris solution	3ml
10% SDS	120µl
10% ammonium persulphate	120µl
TEMED	20µl

Annexure 2.12: 4X Sample buffer

Components	Concentration per 10 ml
Glycerol	4ml
B-mercaptoethanol	2ml
SDS	1.2g
4X stacking Tris	5ml
Bromophenol blue	0.03g

Annexure 2.13: Running buffer

Components	Concentration
Tris HCl	3g
Glycine	14.4g
SDS	1g
Distilled water	1L

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Annexure 2.14: Transfer buffer

Components	Concentration
Tris-base	3.028g
Glycine	14.4g
Methanol	200ml
Distilled water	Up to 1L

Annexure 2.15: ELISA extraction buffer

Components	Concentration (200ml)
$MgCl_2$	5mM
NaCl	1M
$CaCl_2$	5mM
HEPES- free acid	20mM (pH 7.40)
MSF	1mM
Triton X-100	0.01%

3. Results

In this study, the optimal conditions for transforming nodal explants of *Solanum lycopersicum* L. were determined, and a robust protocol for the stable *Agrobacterium-*mediated transformation of tomato cv. Rio Grande with the HPV-18 *L1* gene was established.

Several molecular biology techniques, including conventional PCR and quantitative real-time Polymerase Chain Reaction (qRT PCR), were used to confirm the stable nuclear transformation of the HPV-18 *L1* gene. Finally, to confirm the presence of HPV-18 *L1* protein, dot blot, Western blot, and ELISA were performed.

3.1. Seed Germination

3.1.1. Sterilization of Tomato seeds

Using 0.1% mercuric chloride, tomato seeds were surface sterilized. Seeds were soaked in 0.1% mercuric chloride for 15 seconds. The seeds were washed three times with autoclaved distilled water after being treated with mercuric chloride for three minutes three times. On both the half and full strengths of MS media, the seeds were inoculated and were observed for 30 days.

3.1.2. Germination efficiency of tomato seeds

Sterilized seeds were cultivated in both ½ MS and full MS media under sterile LFH conditions, with the experiment conducted in triplicates and a total of 40 seeds utilized.

Twenty seeds were placed on ½ MS media, and another 20 on full MS media, with each jar containing 6 or 7 seeds. The assessment of germination efficiency was performed 30 days after seed inoculation in both media.

The findings revealed that seeds sterilized with 0.1% HgCl$_2$ on ½ MS media exhibited a notably high germination rate. Although the overall germination efficiency was consistent across both media, standing at 80% as illustrated in Table 3.1 and graphically represented in Figure 3.2, there were variations in the germination rates.

Notably, seeds inoculated on ½ MS media demonstrated a quicker germination compared to the full MS media as depicted in Figure 3.1. Consequently, in subsequent experiments, tomato seeds were specifically inoculated in ½ MS media.
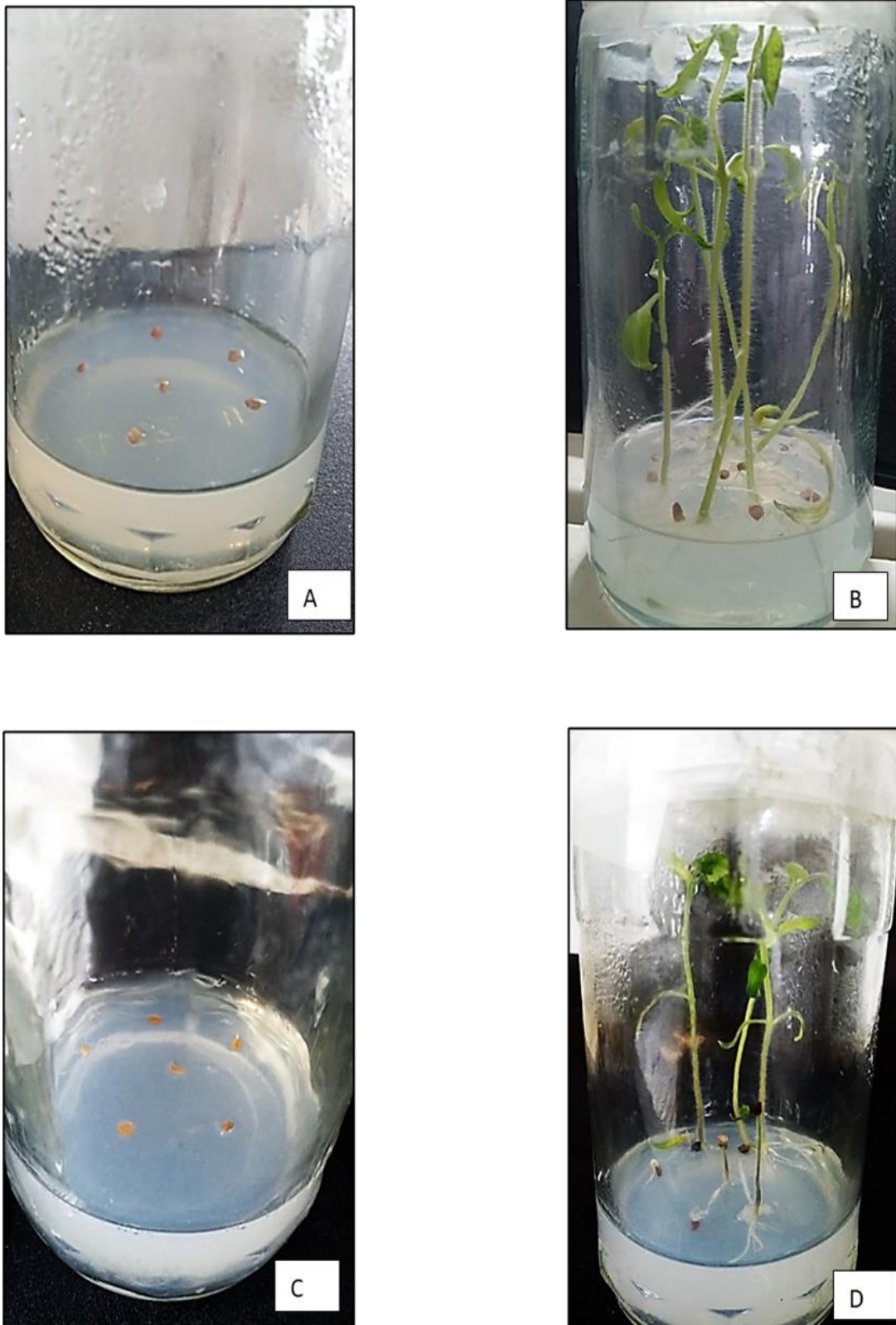
Figure 3.1: Tomato seeds germinated on different strengths of MS media (½ MS and full MS media). (A). Day 1 on ½ MS (B). Day 30 on ½ MS (C). Day 1 on full MS (D). Day 30 on full MS

Table 3.1: Germination efficiency of Tomato seeds on different MS strength

S.No.	MS Strength	Total seeds inoculated	Seeds germinated	Germination efficiency
1	½ MS	20	17	85%
2	Full MS	20	17	85%
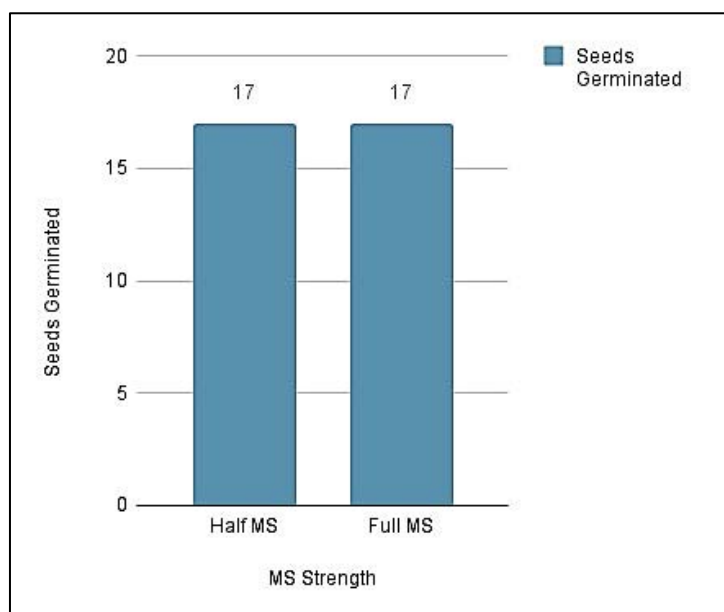
Figure 3.2: Graphical representation of seeds germination efficiency on both MS strengths. The data is from three batches

3.2. Regeneration of *Solanum lycopersicum* L.

Tomato nodal explants underwent regeneration on a carefully optimized regeneration medium (Full MS with vitamins, supplemented with a precise amount of zeatin). The experimental setup involved triplicate trials, and post-inoculation, the explants were monitored for a period of four weeks.

Notably, shoot regeneration was evident in most of the nodal explants, as depicted in Figure 3.3. Initially, callus formation occurred in nearly all nodal explants, followed by subsequent shoot development in most cases.

The efficiency of regeneration for *Solanum lycopersicum* L. nodal explants was calculated after 30 days from the inoculation of explants, and the results are detailed in Table 3.2.

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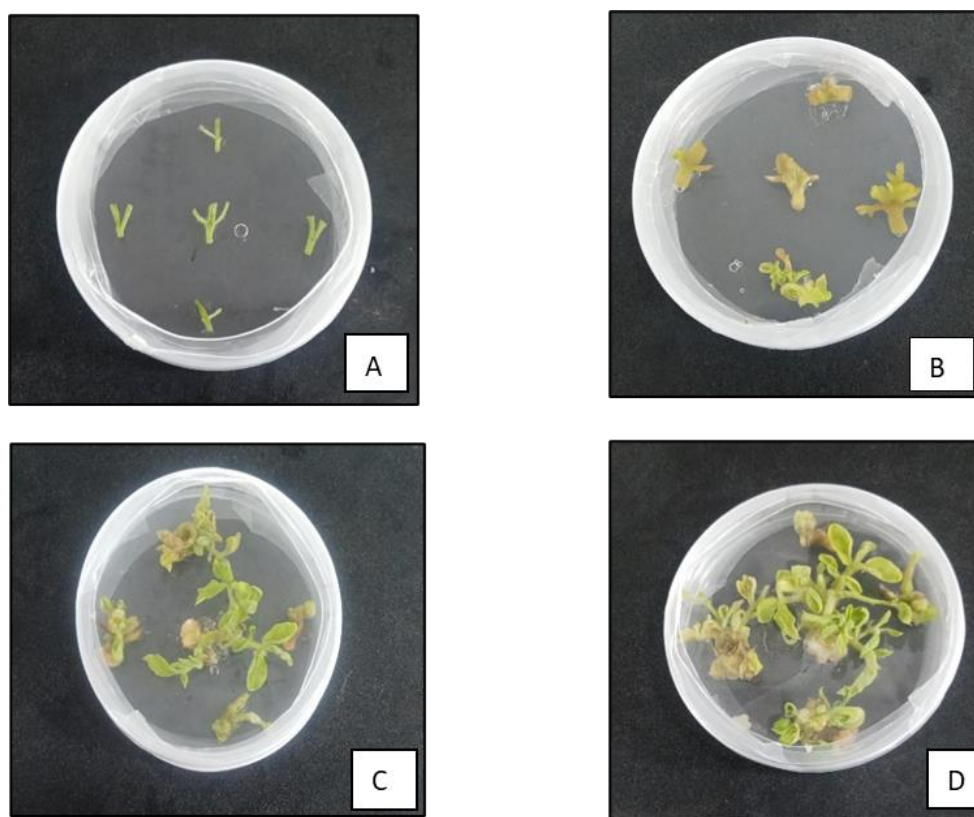
Figure 3.3: Regeneration of tomato nodal explants. (A) Tomato nodal explants at day 1 (B) Tomato nodal explants at day 14 (C) Tomato nodal explants at day 30 (D) Tomato nodal explants after day 30

Table 3.2: Regeneration efficiency of tomato nodal explants

Sr. no	No. of nodes cultivated	No. of nodes regenerated	Regeneration efficiency (%)	Callogenesis efficiency (%)
1	5	4	75%	100%

3.3. Effect of kanamycin concentration on the growth of nodal explants

The effect of kanamycin on the survival of nodal explants was checked. For this, nodal explants were prepared, and they were shifted to two different concentrations of kanamycin to confirm the effect of this antibiotic on these explants. *Solanum lycopersicum* L explants were prepared, and they were inoculated on tomato regeneration media containing Zeatin with two different concentrations of kanamycin i.e., 0 mg/l and 75 mg/l and the results of the following one month were recorded. It was observed that kanamycin halted the regeneration ability of tomato and with the

increase in the concentration of kanamycin, more explants gained the ability to bleach out. 75 mg/l concentration was used as the optimal concentration for bleaching of plants. This concentration of kanamycin was used later for selection of transformed explants. Figure 3.4 shows the effect of different concentrations of kanamycin on tomato explants.
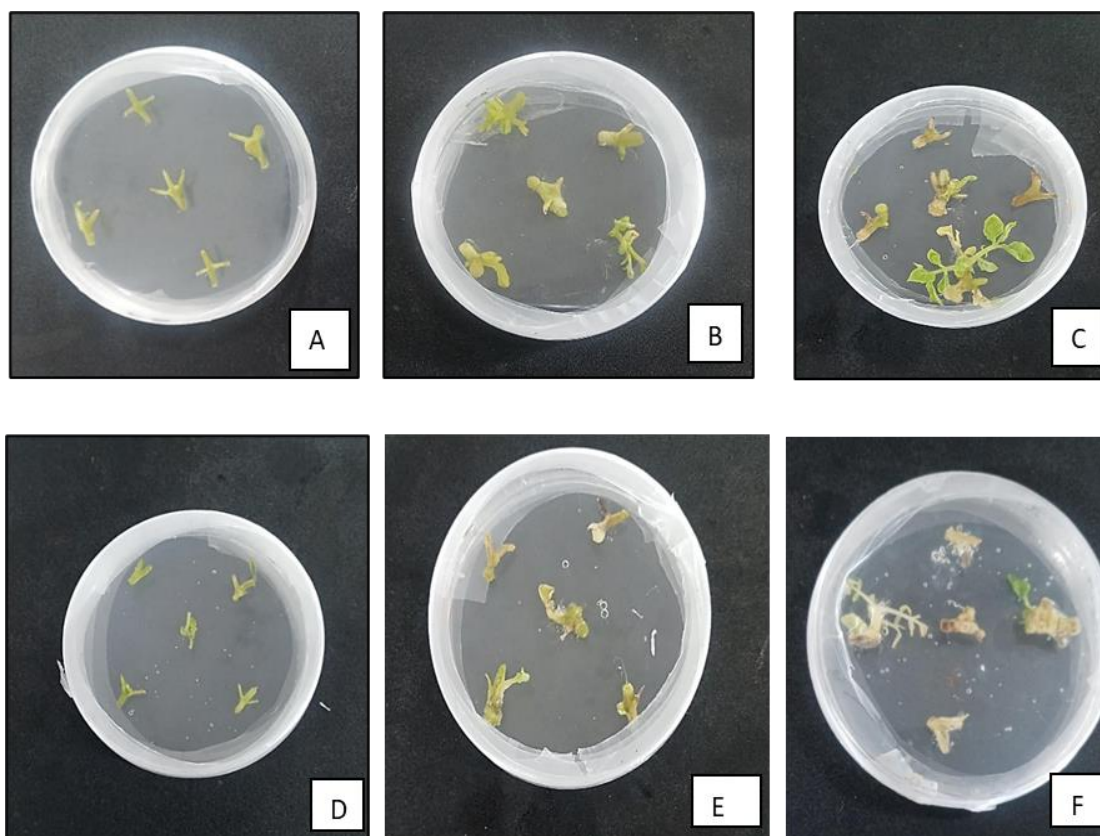
Figure 3.4: Effect of kanamycin concentrations on nodal explants of *Solanum lycopersicum* L. Nodes were inoculated on two different concentrations of kanamycin i.e., 0 mg/l and 75 mg/l and their behavior was observed over the course of one month. (A) Nodes at 0 mg/l kanamycin at day 1, (B) Nodes at 0 mg/l kanamycin at day 15, (C) Nodes at 0 mg/l kanamycin at day 30, (D) Nodes at 75 mg/l kanamycin at day 1, (E) Nodes at 75 mg/l kanamycin at day 15, (F) Nodes at 75 mg/l kanamycin at day 30

3.4. Transformation

The HPV-18 *L1* antigen 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 this gene (in pGWB5 binary vector) via *Agrobacterium*-mediated

transformation. Integration of the HPV-18 *L1* antigen 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 HPV-18 *L1* gene

3.4.1.1 PCR confirmation of plasmid containing HPV-18 *L1* gene by using nuclear primers

Sambrook and Rusell (2001) protocol of plasmid isolation was used. The presence of HPV-18 *L1* antigen in plasmid was confirmed by conventional PCR by using two different types of primers for the amplification of transgene. The gel results showed the presence of transgene in the isolated DNA samples when PCR was done by using HPV-18 *L1* nuclear forward and nuclear reverse primers.
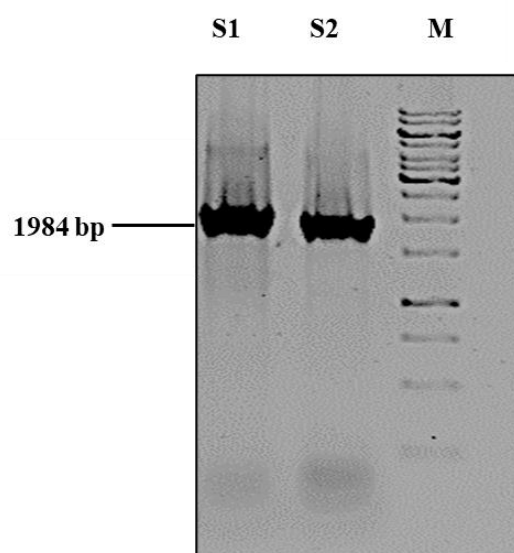
Figure 3.5: PCR confirmation of HPV-18 *L1* plasmid with nuclear primers. (S1) PCR product of sample 1 (1984 bp), (S2) PCR product of sample 2 (1984 bp), (M) 1kb Marker

3.4.1.2. PCR confirmation of plasmid containing HPV-18 *L1* gene by using internal primers

The presence of HPV-18 *L1* antigen in plasmid was confirmed by conventional PCR by using two different types of primers i.e. nuclear and internal set of primers for the amplification of transgene. The gel results showed the presence of transgene in the isolated DNA samples when PCR was done by using HPV-18 *L1* internal forward and

internal reverse primers as shown in figure 3.6, where PCR products sample 1 and sample 2 shows a fragment of 372bp length.
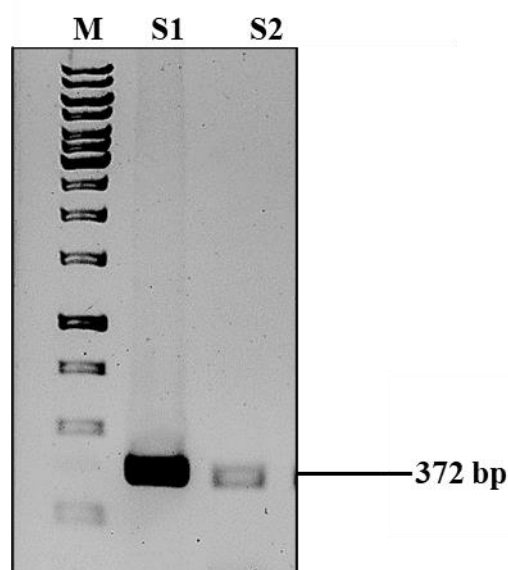
Figure 3.6: PCR confirmation of HPV-18 *L1* plasmid with internal primers. (M) 1 kb Marker. PCR product of Sample 1 (372 bp). (S2) PCR product of Sample 2 (372 bp)

3.5. *Agrobacterium*-mediated transformation

3.5.1. *Agrobacterium*-mediated stable transformation of *Solanum lycopersicum* L.

Nodes of *Solanum lycopersicum* L were used for transformation. Explants were prepared by cutting them into smaller pieces. *Agrobacterium tumefaciens* with pGWB5 containing HPV-18 *L1* gene was used to infect these nodal explants. After infection, the nodes were shifted to co-cultivation plates before their transfer to kanamycin plates.

3.5.1.1. Optimization of infection time

In this experiment, nodes were categorized into two groups, and both groups underwent infection with *Agrobacterium*. One set of nodes received treatment with the bacterial culture for 8 minutes, while the other set was treated for 10 minutes. The explants were closely observed over several days, as depicted in Figure 3.7.

It was observed that as the infection time increased, the removal of excess bacteria became more challenging, and the efficiency of regeneration or transformation of nodes diminished. Consequently, an infection time of 8 minutes was identified as the

optimal duration for the transformation of nodal explants. Results regarding the infection time are tabulated in Table 3.3 and show 60% regeneration efficiency in nodes infected for 8 minutes.

Table 3.3: Effect of infection time on regeneration of explants

Infection time	Total no. of nodes	Nodes regenerated	Regeneration efficiency (%)
8 minutes	5	3	60%
10 minutes	5	1	20%

3.5.1.2. Optimization of co-cultivation time

Explants were placed on co-cultivation media after infection and kept in the dark for two to three days. The explants were examined following the corresponding co-cultivation period. When the co-cultivation time was extended, it was found that nodes' capacity for regeneration reduced.

Explants that were inoculated for two days exhibited a higher capacity for regeneration. The results for optimization of co-cultivation time are tabulated in Table 3.4 and figure 3.8. Based on the results, it was determined that an infection period of 8 minutes and a co-cultivation period of 2 days was the ideal period for nodal explants to transform effectively. Thus, the *Solanum lycopersicum* L. nodal explants were transformed under these ideal conditions.
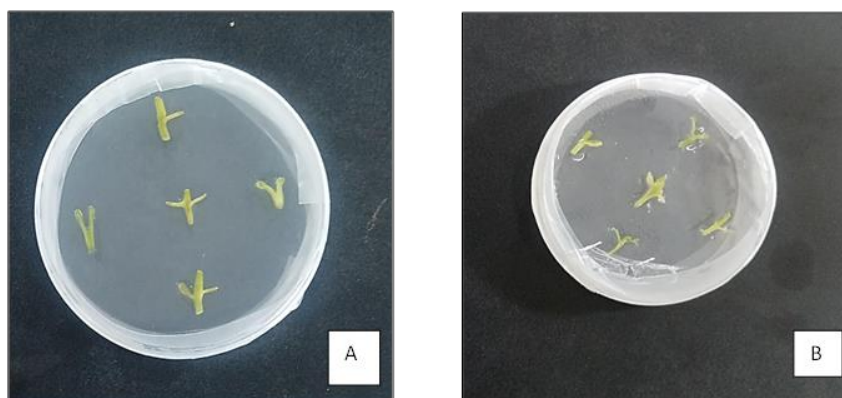
Figure 3.7: (A) Nodal explants after 2 days co-cultivation and 8 minutes infection time. (B) Nodal explants after 3 days co-cultivation time and 10 minutes infection

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Table 3.4: Effect of co-cultivation time on the regeneration efficiency of nodes

Co-cultivation time	Total No. of nodes	No. of nodes regenerated	Regeneration efficiency (%)
2 days	5	3	60%
3 days	5	1	20%
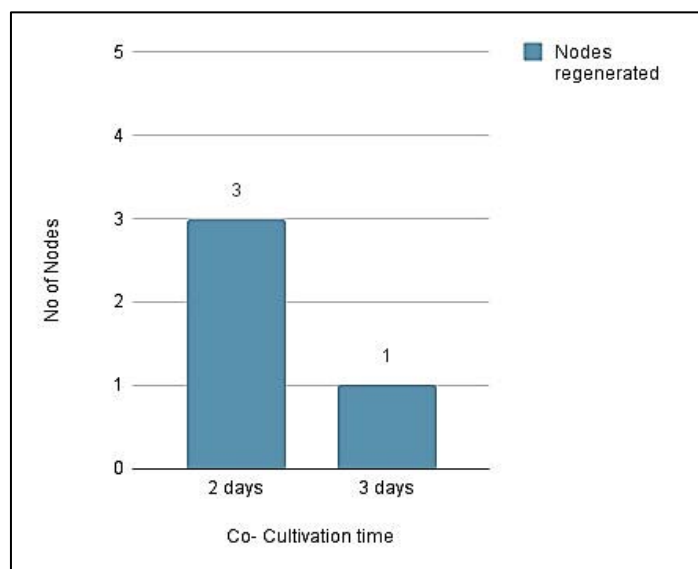
Figure 3.8: Graphical representation of the effect of co-cultivation time on regeneration or transformation efficiency of nodal explants

3.5.1.3. Stable Transformation

After co-cultivation, plants were shifted to the selection media containing 75mg/l kanamycin. Following the meticulous optimization of various factors, *Agrobacterium*-mediated transformation was conducted under refined conditions. Nodes underwent infection with *Agrobacterium* carrying the HPV-18 *L1* gene for a duration of 8 minutes.

Subsequently, the treated samples were kept in the dark for a 2-day co-cultivation period. After this period, the explants were washed with liquid ½ MS containing cefotaxime to eliminate excess bacteria and were then transferred to selection plates containing kanamycin. This set of experiments, designated as batch 1 under optimized conditions, was compared with batch 2, which involved a 3-day co-cultivation time.

Figure 3.9 provides a visual comparison of the transformation outcomes between batch 1 with optimized conditions and batch 2 with an extended co-cultivation time. The transformation efficiency was determined to be 60% in batch 1 and 20% in batch 2.

Batch 1   Batch 2

Batch 1   Batch 2
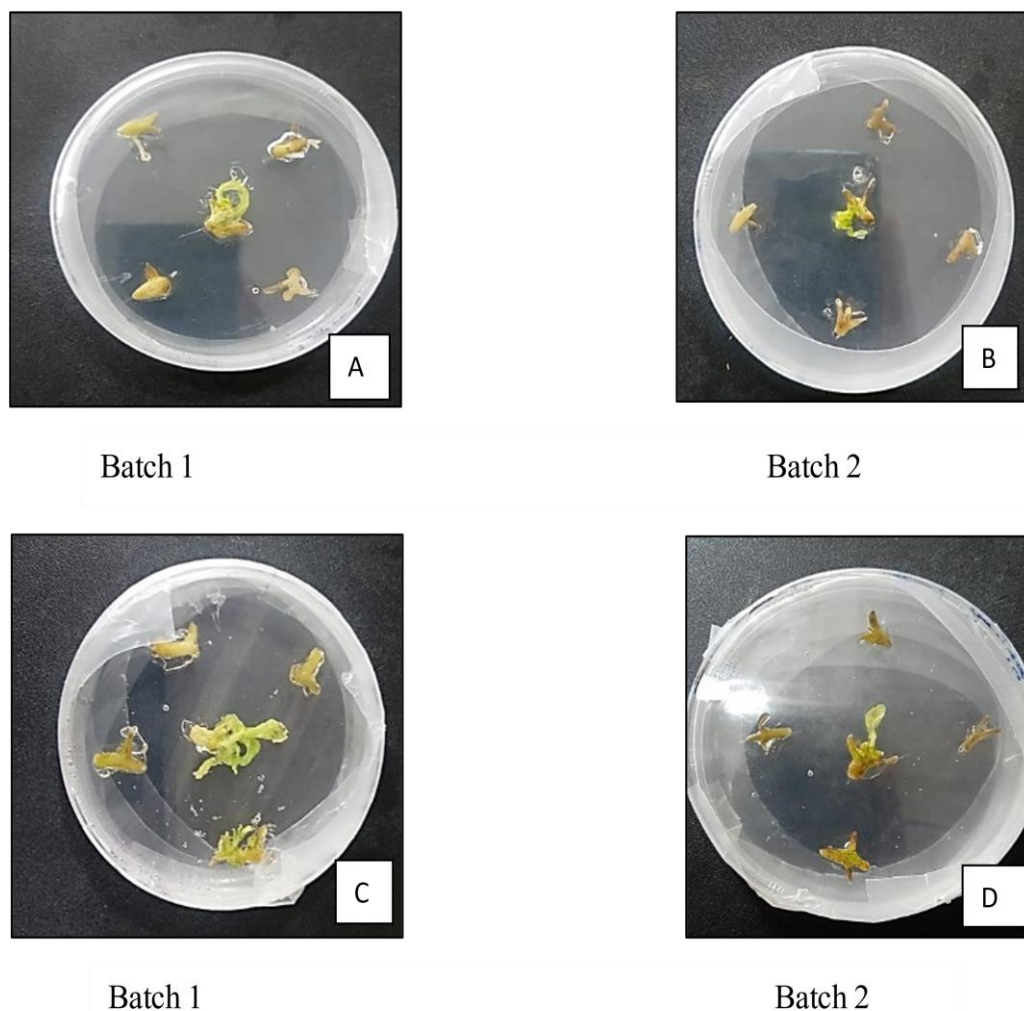
Figure 3.9: Transformed explants on selection plates. (A & B) Transformed explants on selection plates after 2 weeks. (C & D) Transformed explants on selection plates after 4 weeks.

3.6. Confirmation of transformation using molecular biology techniques

Nodal explants were transformed with HPV-18 *L1* gene in pGWB5 binary vector by *Agrobacterium*-mediated transformation. The integration of HPV-18 *L1* gene into the plant genome of *Solanum lycopersicum* L was confirmed by using conventional PCR and qRT- PCR. Furthermore, to confirm the protein in transformed nodal explants dot

blot and western blotting was done. The results of all these techniques are illustrated with figures in below section.

3.6.1. PCR confirmation of transgene by using different types of HPV-18 *L1* primers

Genomic DNA from the plant was extracted using the CTAB method, following the protocol outlined by Murray and Thompson (1980). Prior to PCR confirmation, the extracted DNA underwent validation through agarose gel electrophoresis, as illustrated in Figure 3.10.

The confirmation of the presence of the HPV-18 *L1* gene in *Solanum lycopersicum* L. was achieved through conventional PCR, utilizing specific primers designed for transgene amplification.

Specifically, HPV-18 *L1*-nuclear forward and reverse primers, along with HPV-18 *L1* internal forward and reverse primers, were employed for the verification of the HPV-18 *L1* transgene. The PCR product was then loaded onto an agarose gel to confirm the presence of the targeted gene. Figure 3.11 depicts the gel image showcasing amplified bands of 1984bp with HPV-18 *L1*-nuclear primers and Figure 3.12 shows gel image of bands of 372 bp with HPV-18 *L1* internal primers, providing conclusive evidence of the HPV-18 *L1* transgene. Consequently, the successful transformation of *Solanum lycopersicum* L. cv. Rio Grande plants was validated.
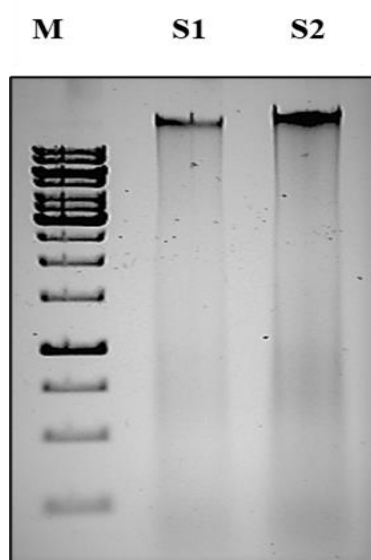
Figure 3.10: Confirmation of DNA isolated from plant samples.1 kb marker (M), Sample 1 (S1), Sample 2 (S2)

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3.6.1.1. PCR confirmation of transgene by using HPV-18 *L1* nuclear primers.
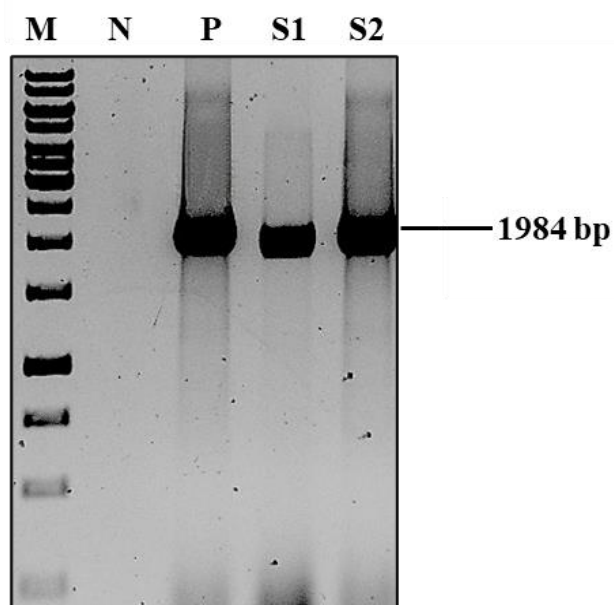
Figure 3.11: PCR confirmation of transgene using nuclear primers. (M) 1 kb marker, (N) Negative control, (P) PCR product of Positive control, 1984 bp, (S1) PCR product of Sample 1, 1984 bp, (S2) PCR product of Sample 2, 1984 bp

3.6.1.2. PCR confirmation of transgene by using HPV-18 *L1* internal primers
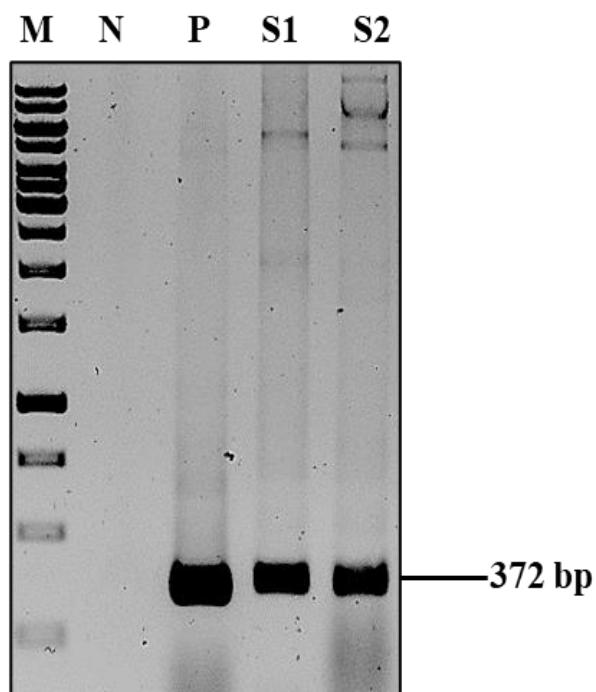
Figure 3.12: PCR confirmation of transgene using internal forward and internal reverse primers. (M) 1 kb marker, (N) Negative control, (P) PCR product of Positive

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control, 372 bp, (S1) PCR product of Sample 1, 372 bp, (S2) PCR product of Sample 2, 372 bp

3.6.2. Calculation of HPV-18 *L1* copy number in transgenic *Solanum lycopersicum* L by qRT-PCR

The Schmittgen and Livak (2008) protocol was followed to determine the copy number of all transformed samples. In PCR-positive samples, the copy number of the transgene (HPV-18 *L1*) was determined by qRT-PCR. β actin, an endogenous gene, was also employed. The standard curve was plotted using the Cq values of the transgenic and the endogenous gene (table 3.5), which gave the correlation coefficient values for the two genes, respectively. The rline was calculated using SQ values and gave us the copy number of the transgene by applying the formula.

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

Table 3.5: Average Cq values of transgene vs endogenous gene

Sample	Average Cq values for HPV-18 *L1* transgene	Average Cq values for β actin
HPV18-1	25.9335982	29.82290853
HPV18 -1:10	36.43070594	31.82694862
HPV18 -1:100	40.09867171	32.77485378
HPV18-1:1000	41.62329885	33.31442407

This provided the correlation coefficient values for transgene HPV-18 *L1* and β actin. All these values were used to calculate the copy number for HPV-18 *L1* gene which was equal to **1**.

3.6.3. Dot Blot

Dot blot was performed to check the presence of protein within the samples. Specific antibodies were used to check for HPV-18 *L1* protein. Samples, both wild type as well as protein containing samples were loaded on the blot.

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Positive results were observed for both the samples unlike wild-type sample. Hence this confirmed the presence of transgenic protein within our samples. Figure 3.13 shows the results of dot blot.
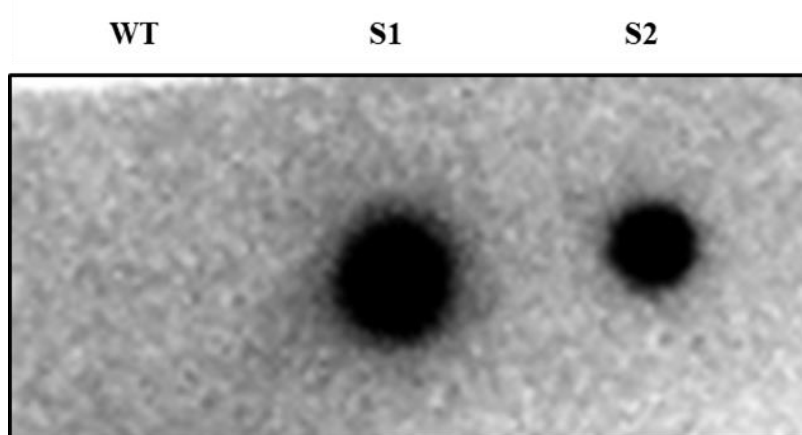
Figure 3.13: Confirmation of presence of HPV-18 *L1* protein in transformed samples by Dot Blot. Wild type (WT), sample 1 (S1), sample 2 (S2)

3.6.4. Western blotting

Western blotting was used to check the expression of HPV-18 *L1* protein. The monomeric form of HPV-18 *L1* protein with size equal to 56.5 kDa is shown in figure 3.14. By using specific antibodies, protein was confirmed in the transgenic samples with no band observed for wild-type sample (figure 3.14). This detection of HPV-18 *L1* protein confirmed the successful stable transformation of tomato nodes.
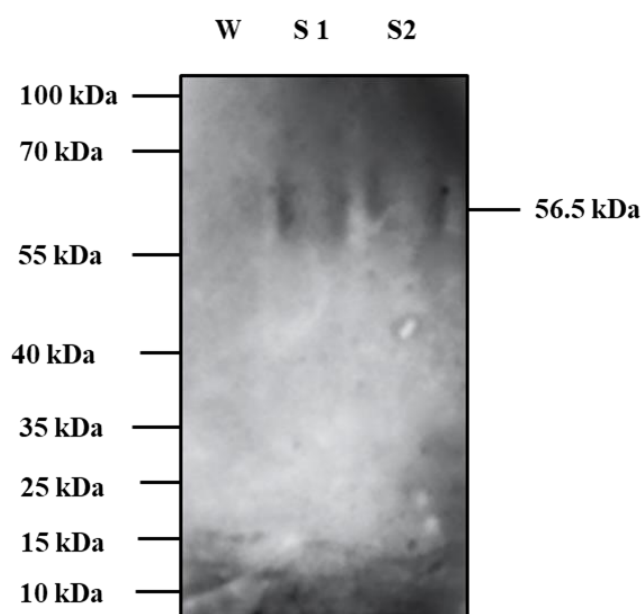
Figure 3.14: Confirmation of HPV-18 *L1* protein by Western blotting. Wild type (W). Transformed sample 1 (S1). Transformed sample 2 (S2)

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3.6.5. ELISA

Indirect ELISA was used to confirm the presence of HPV-18 *L1* antigen within our transgenic samples. Total soluble protein extracted from transgenic plants was used. The data presented in figure 3.15 shows an increased absorbance of transgenic samples compared to the wild type. Among the two samples, S1 shows the highest absorbance which means that it contains the highest amount of antigen.
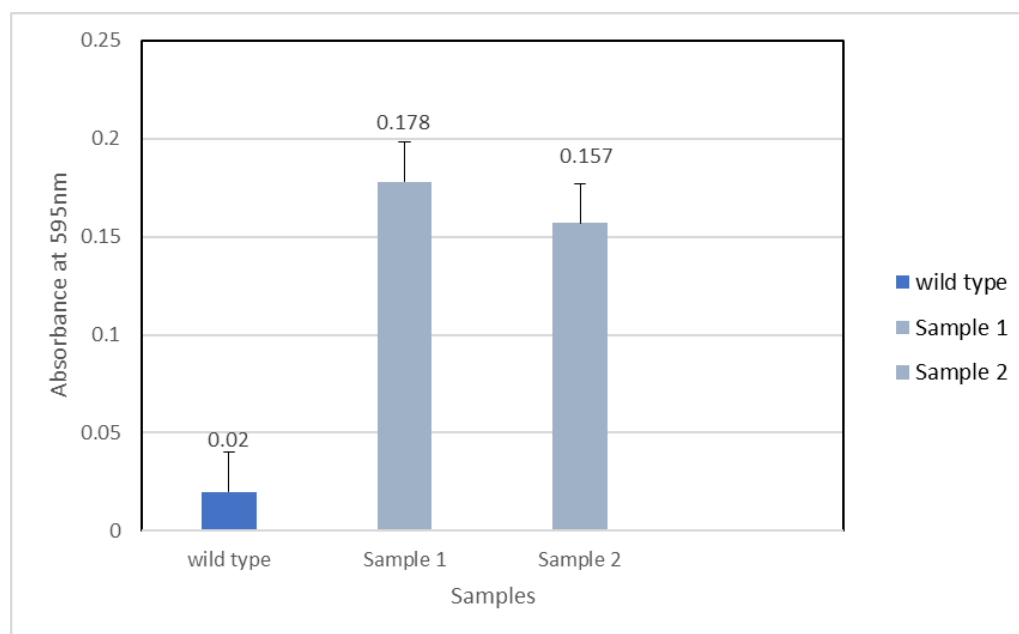
Figure 3.15: Graphical representation of ELISA of wild-type sample and transgenic tomato samples. S1 and S2 are two transgenic nodal samples

4. Discussion

Human papillomavirus (HPV) is recognized as the predominant cause of cervical cancer worldwide. Recent studies emphasize that an estimated 99.7% of cervical cancer cases are attributed to persistent high-risk genital HPV infections (Bray *et al.,* 2018). The intricate link between high-risk HPV strains and the progression from low to high-grade cervical intraepithelial neoplasia, culminating in invasive disease, has been increasingly documented. Among the extensive array of HPV types, HPV-16 and HPV-18 stand out as the most prevalent and infectious, collectively responsible for approximately 70% of cervical cancers globally (Bednarczyk *et al.,* 2011). Notably, ongoing research suggests a nuanced understanding of the molecular mechanisms by which these specific HPV strains contribute to carcinogenesis. In response to this public health challenge, three prequalified vaccines against cervical cancer are currently available in the market. Two of these vaccines offer exclusive protection against HPV-16 and HPV-18, targeting the majority of cervical cancer cases (Roden *et al.,* 2006). The third vaccine provides additional coverage against five other oncogenic HPV types, addressing the remaining 30% of cervical cancers.

The World Health Organization (WHO) has devised a comprehensive strategy aimed at eliminating cervical cancer globally by 2030. The strategy encompasses multifaceted goals, including widespread vaccination campaigns targeting individuals before or by the age of 15, routine screening at ages 35 and 45, and ensuring pre-cancer treatment for 90% of identified cases (Sung *et al.,* 2021). This strategic approach underscores the importance of a holistic and proactive stance towards cervical cancer prevention and control.

The limitations of current VLP-based vaccines against human papillomavirus (HPV) in terms of therapeutic application and cross-protection have spurred the exploration of alternative approaches. Furthermore, they are costly, in terms of their pre- and post-production charges, and availability to the people of low-income countries as they cannot afford the travel and cold chain maintenance of these vaccines (Fernandez *et al.,* 2016). A promising avenue of research involves the development of plant-based vaccines using subunit capsomere protein *L1* with an adjuvant LTB. This approach has demonstrated a notable ability to induce an antigenic response. Plant-based vaccines offer a cost-effective alternative to traditional vaccines, addressing the

accessibility challenges faced by low-income nations. These vaccines are not only economically viable but also safe, easily scalable, and amenable to large-scale production. Additionally, their mode of administration, particularly the oral route, represents a convenient and patient-friendly approach.

The current study focuses on the stable transformation of the HPV-18 *L1* antigen from Human Papillomavirus into *Solanum lycopersicum* L. (tomato) via *Agrobacterium*-mediated transformation for developing a plant-based edible vaccine against cervical cancer.

4.1. Human papillomavirus antigen expression in plants

Recent advancements in plant biotechnology and molecular biology have facilitated the production of subunit vaccines using plants as bioreactors. However, the practical utility of these systems initially faced limitations due to associated post-production costs (Rybicki, 2014). Over the past decade, the expression of human papillomavirus (HPV) antigens in plants has garnered substantial attention (Jordan *et al.,* 2019). The development of a cost-effective prophylactic vaccine against oncogenic HPV types holds significant promise for reducing the global burden of cervical cancer. Notably, various plant species have been employed for expressing HPV capsid proteins, virus-like particles (VLPs), and chimeras. In 2005, HPV-16 *L1* protein was successfully expressed in the nuclear genome of *Nicotiana tabacum* (Liu *et al.,* 2005). Furthermore, nuclear expression of the HPV-16 *L1* capsid protein in *Arabidopsis thaliana* was achieved in 2007

4.2. Use of Tomato as a model plant

Solanum lycopersicum L., commonly known as tomato, stands as one of the most extensively cultivated crops worldwide. Its relatively small genome size contributes to a swift cultivation process, making it an expeditious choice for agricultural endeavors. The inherent property of cross-compatibility renders it particularly advantageous for genetic studies. These distinctive features position the tomato as an optimal candidate for expressing foreign proteins and vaccine production (Matteo *et al.,* 2011).

In the realm of genetic engineering, Tomato (*Solanum lycopersicum* L.) assumes prominence as a model plant owing to its nutritional significance and various favorable attributes. These include a compact and small genome, a brief life cycle,

and the capability for self-fertilization (Chetty *et al.,* 2013). These collective characteristics underscore the utility of tomato as a valuable resource in scientific research, especially in the context of genetic manipulation and molecular studies.

4.3. Sterilization and Germination of Tomato Seeds

In the context of seedling generation within a tissue culture experiment, the crucial step of surface sterilization is imperative to mitigate contamination risks. In the present investigation, preceding the transformation process, seed sterilization and germination of tomato seeds, specifically cv. Rio Grande, were conducted using a 0.1% mercuric chloride ($HgCl_2$) solution for 15-20 seconds on half MS media, yielding a germination efficiency of 80%. The observed germination rate was influenced by the duration of exposure to mercuric chloride, potentially attributable to its bleaching properties. Earlier research employed three sterilizing agents: mercuric chloride, sodium hypochlorite, and hydrogen peroxide to sterilize tomato seeds, revealing that 5% sodium hypochlorite for 15 minutes resulted in the highest germination efficiency (Bharti *et al*., 2018)

4.4. Tomato plant regeneration

The selection of phytohormones, both in terms of type and concentration, is a critical determinant in regulating shoot and root induction during the regeneration of tomato explants. In this investigation, the application of 2mg/L zeatin as a regeneration hormone, in conjunction with full MS media enriched with vitamins, proved effective for the regeneration of tomato nodal explants. The outcomes demonstrated 100% callogenesis efficiency and a 75% shoot regeneration efficiency. Additionally, rooting was observed on the same media after a 30-day period. Notably, zeatin exhibited greater efficacy compared to BAP (6-benzylaminopurine) for tomato explants regeneration. Earlier research also employed zeatin as a regeneration phytohormone for hypocotyl and cotyledonary explants of tomato (Vikram *et al.,* 2012).

4.5. Stable *Agrobacterium*-mediated transformation

Efficient and stable *Agrobacterium*-mediated transformation relies on various factors influencing its effectiveness, including bacterial inoculum density, infection time, and co-cultivation duration. The selection of optical density (OD) is a critical determinant of transformation efficiency. In this study, an OD range of 0.6 to 0.8 at 600nm was

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identified as optimal for successful transformation. Higher OD values exceeding 0.8 were found to have detrimental effects on explants, leading to a reduction in both *Agrobacterium* infection efficiency and overall transformation success. Notably, an OD of 0.6 at 600nm emerged as the most favorable for achieving stable transformation in tomatoes using *Agrobacterium* carrying HPV-18 *L1*.

In the present study, optimization of infection time and co-cultivation time for *Solanum lycopersicum* L. was conducted. Two distinct infection durations, namely 8 minutes and 10 minutes, were assessed. Explants subjected to 8 minutes infection period exhibited superior regeneration, achieving 60% regeneration efficiency. In contrast, those infected for 10 minutes displayed lower regeneration rates, reaching only 20% efficiency. Consequently, the 8 minutes infection duration emerged as the optimal timeframe for achieving successful transformation of tomato cv. Rio Grande with *Agrobacterium*. These findings align with results reported by Lin *et al.* (2016).

In the examination of co-cultivation time, tomato explants infected with *Agrobacterium* containing HPV-18 *L1* were subjected to co-cultivation periods of 2 and 3 days. Notably, explants exposed to a 2-day co-cultivation exhibited higher transformation or regeneration efficiency compared to those co-cultivated for 3 days. The optimal co-cultivation duration for achieving stable transformation in tomatoes was determined to be 2 days. Similar findings were reported by Opabode (2006). The study observed that prolonged co-cultivation and infection times led to excessive bacterial growth, negatively impacting plant growth. Additionally, the introduction of acetosyringone at a concentration of 200μM in both co-cultivation and infection media enhanced transformation efficiency. To mitigate bacterial growth, cefotaxime was identified as the most effective antibiotic for *Agrobacterium,* with concentrations of 500mg/l in washing media and 250mg/l in selection media being employed.

To confirm the integration and expression of the transgene, a range of molecular techniques was employed, including PCR, Dot Blot analysis, qRT-PCR, Western Blot, and ELISA. Specifically, for the stable integration of the transgene in transformed tomato explants, PCR was conducted using specific primers, with wild-type explants serving as the control. The presence of 1984bp PCR fragments using HPV-18 *L1* nuclear primers and 372bp PCR fragments with HPV-18 *L1* internal primers provided

confirmation of the HPV-18 *L1* gene's presence in the transformed tomato explants. Notably, no corresponding bands were observed in the wild-type samples.

Following the methodology outlined by Wen *et al.* (2012), qRT-PCR was employed to ascertain the copy number. Transgenic samples exhibited positive results, indicating the presence of the HPV-18 *L1* gene, while no results were observed in the wild type samples. The determined copy number for the HPV-18 *L1* gene was 1.

To analyze the presence of the HPV-18 *L1* protein, both Dot blot and Western Blot techniques were employed. In the Dot blot, a Chemiluminescence signal was observed in the total soluble protein extracted from transgenic samples, whereas no signal was detected in the case of the wild type. Similarly, in the Western Blot, total soluble protein was extracted, subjected to PAGE, and the resulting bands were transferred to a nitrocellulose membrane. Following treatment with primary and secondary antibodies, and subsequent exposure to substrate, the blot was visualized. A distinct 56.5kDa band was evident in the stably transformed protein sample, while no corresponding band was observed in the wild type. The presence of the 56.5 kDa band confirmed the expression of the HPV-18 *L1* protein in the transgenic samples of tomato plants.

ELISA was conducted to quantify the antigenic protein in the samples. Stably transformed explants' protein samples exhibited a noticeable color change upon the addition of the TMB substrate, distinguishing them from blank and wild-type samples. This color change conclusively confirmed the presence of the HPV-18 *L1* antigen in the transgenic samples. Notably, it was observed that the S1 transgenic sample displayed a higher absorbance at 595 nm compared to S2, suggesting that S1 contained a higher concentration of the antigen.

4.6. Conclusion and future perspectives

In this study, various parameters crucial for achieving stable *Agrobacterium*-mediated transformation of *Solanum lycopersicum* L. were systematically tested and optimized.

- The seeds of Tomato cv. Rio Grande were effectively sterilized by subjecting them to a treatment with 0.1% HgCl2 for 15-20 seconds. Subsequently, these sterilized seeds demonstrated efficient germination when placed on half MS media.

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- Regeneration of nodal explants from Tomato cv. Rio Grande can be successfully accomplished by using full MS with vitamins, supplemented with 2mg/l Zeatin.

- Optimal parameters such as co-cultivation duration, infection time, and OD600 of the *Agrobacterium* culture were optimized to achieve successful and stable transformation of tomatoes.

The present study offers meticulously optimized conditions for achieving stable transformation in tomato with HPV-18 *L1*. Maximum transgene expression was realized through a strategic combination of 2 days of co-cultivation following an 8 minute infection period of nodal explants from tomatoes. Collectively, these findings lay a foundation for potential applications, notably in the development of a cost-effective subunit vaccine against cervical cancer, with the prospect of oral delivery. Furthermore, this research serves as a valuable resource for refining and streamlining a reliable procedure for stable *Agrobacterium*-mediated transformation in diverse tomato cultivars, employing the recommended optimum conditions for various variables.

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