# Development of Plant-Based Subunit Vaccine Against Vibriosis for Use in Aquaculture

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**2024**

# Development of Plant-Based Subunit Vaccine Against Vibriosis for Use in Aquaculture

*A dissertation in the partial fulfillment of the requirements for the degree*

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*Submitted by*

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**2024**

بسم الله الرحمن الرحيم

**This thesis is dedicated**

**To**

**My parents**

**I would like to acknowledge my debt to my loving parents whose wishes motivate me to strive for higher education. I attribute all my success in life to the moral, intellectual and physical education I received from them. Without their understanding and continual support, it would have been impossible for me to finish this work.**

**And**

**To**

**My Wife**

**Who believes in the richness of learning. As I believe that the best kind of knowledge is what is learned for its own sake.**

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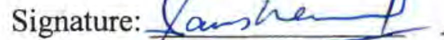
4. **Chairperson:** Signature: 
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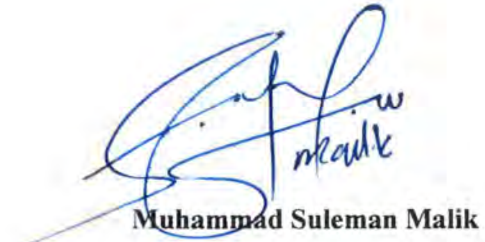

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| °C | Degree Centigrade |
| µL | Microliter |
| µm | Micrometer |
| 2D | Two-Dimensional |
| 3D | Three-Dimensional |
| Ab | Antibodies |
| ACC | Automated Cross-Covariance |
| AI | Aliphatic Index |
| Amp | Ampicillin |
| ANN | Artificial Neural Networks |
| APCs | Antigen Presenting Cells |
| APS | Ammonium Persulfate |
| ARGs | Anti-Biotic Resistant Genes |
| BAP | 6-Benzylaminopurine |
| BCE | B-Cell Epitopes |
| BCR | B-Cell Antigen Receptor |
| Bp | Base Pair |
| BSA | Bovine Serum Albumin |
| Cm | Centimeter |
| CO | Codon Optimized Sequence |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| CTLs | Cytotoxic T-Cells |
| Cv | Cultivar |
| DCs | Dendritic Cells |
| DNA | Deoxyribose Nucleic Acid |
| *E. Coli* | *Escherichia Coli* |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| F | Forward |

| | |
|---|---|
| FAO | Food And Agriculture Organization |
| FDA | US Food and Drug Administration |
| FFT | Fast Fourier Transform |
| G | Gram |
| GMOs | Genetically Modified Organisms |
| GoI | Gene of Interest |
| GRAVY | Grand Average of Hydrophobicity |
| HB | Hydrogen Bonds |
| His | Histidine Residue |
| HLA | Human Leukocyte Antigen |
| HRP | Horse Radish Peroxidase |
| Hyg | Hygromycin |
| I | Internal |
| IAA | Indole Acetic Acid |
| ICs | Immune Complexes |
| Ig | Immunoglobulin |
| IUIS | International Union of Immunological Societies |
| Kan | Kanamycin |
| Kb | Kilobytes |
| kDa | Kilo Dalton |
| LA | Luria Agar |
| LB | Luria Broth |
| LFH | Laminar Flow Hood |
| LIC | Ligation Independent Cloning |
| Lm | *Listeria Monocytogenes* |
| M | Molar |
| M | Meter |
| mAb | Monoclonal Antibody |
| mg | Milligram |
| mg/mL | Milligram/Milliliter |

| | |
|---|---|
| mg/mol | Milligram/Mole |
| MHC | Major Histocompatibility Complex |
| MLSA | Multi Locus Sequence Analysis |
| mM | Millimole |
| MS | Murashige And Skoog |
| MW | Molecular Weight |
| NaOH | Sodium Hydroxide |
| NCBI | National Center for Biotechnology Information |
| Ng | Nanograms |
| NI | Non-Infiltrated |
| NIAID | National Institute of Allergic and Infectious Diseases |
| Nm | Nanometer |
| *npt*II | Neomycin Phosphotransferase II |
| OD | Optical Density |
| $OD_{600}$ | Optical Density At 600nm |
| OmpK | Outer Membrane Protein K |
| OMPs | Outer Membrane Proteins |
| OS | Original Sequence |
| OVC | OmpK vaccine Construct |
| P. rank | Percentile Rank |
| PAMPs | Pathogen-Associated-Molecular Patterns |
| PCR | Polymerase Chain Reaction |
| pH | Power of Hydrogen Ions |
| pI | Theoretical Isoelectric pH |
| PMSF | Polymethyl-Sulfonyl-Fluoride |
| PP | Peyer’s Patches |
| PRRs | Pattern-Recognition Receptors |
| psi | Pounds Per Square Inch |
| PTGS | Post-Transcriptional Gene Silencing |
| QM | Quantitative Matrices |

| | |
|---|---|
| qRT-PCR | Quantitative Real-Time PCR |
| R | Reverse |
| Rif | Rifamycin |
| rDNA | Recombinant DNA |
| RICs | Recombinant Immune Complexes |
| RMOP | Revised Medium for Organogenesis of Plants |
| RNA | Ribonucleic Acid |
| Rpm | Revolutions Per Minute |
| RPS | Relative Percent Survival |
| RV | Reverse Vaccinology |
| SDS | Sodium Dodecyl Sulphate |
| SOC | Super Optimal Broth with Catabolite Repression |
| Sp | Species |
| Spp | Several Species |
| subsp | Subspecies |
| SV | Structural Vaccinology |
| SVM | Support Vector Machines |
| T-DNA | Transfer Deoxyribose Nucleic Acid |
| Tet | Tetracycline |
| Th-cells | T-Helper Cells |
| Ti | Tumor Inducing |
| TLP | Total Leaf Protein |
| TLRs | Toll-Like Receptors |
| Tm | Melting Temperature |
| TMB | 3,3,5,5-Tetramethylbenzidine |
| TP | Transgenic Protein |
| TSP | Total Soluble Protein |
| USDA | United States Department of Agriculture |
| UV | Ultraviolet |
| V | Volt |

| | |
|---|---|
| var. | Variety |
| VdW | Van Der Waal's Forces |
| Ver | Version |
| Vir | Virulence |
| VLPs | Virus-Like Particles |
| VNPs | Virus-Based Nanoparticles |
| WCI | Whole Cell Inactivated |
| WHO | World Health Organization |
| WT | Wild Type |
| A | Alpha |
| β | Beta |

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**Muhammad Suleman Malik**

# ABSTRACT

## ABSTRACT

Vibriosis is caused by *Vibrio anguillarum* in various species of aquaculture. Vibriosis is a deadly hemorrhagic septicemic disease that affects fish, bivalves, and crustaceans, causing considerable economic losses. There are different hurdles to controlling vibriosis such as the lack of effective vaccines, their reversion to virulent form from attenuated ones, and the development of resistance to existing antibiotics due to overuse. Secondly, for fish vaccination, methods such as injection and immersion are costly. The injection method requires expensive machinery and skilled labour while immersion vaccination requires multiple doses and a large amount of vaccine. The alternate method for fish vaccination is oral vaccination. This method is stress-free and cost-effective. The vaccine antigen expressed in plants could be used to develop a cost-effective oral vaccine as the plant-based expression system has several advantages like ease of cultivation in large areas, considerably high yield of antigens, easy storage at room temperature and low cost.

A novel, secure, and stable vaccine is needed to eradicate vibriosis. In the present study, for reverse vaccinology and consequent plant-based expression, the outer membrane protein K (OmpK) of *V. anguillarum* was chosen. The OmpK is conserved among many *Vibrio* species, thus a good candidate for broad spectrum vaccine against vibriosis. Bioinformatics tools were used to investigate the biochemical and physiochemical characteristics of the OmpK vaccine construct (OVC). OVC proved as an ideal vaccine candidate as it demonstrated immunogenic, non-allergic, and non-toxic behaviors. Other physiochemical characteristics were also satisfying and supported the OVC as a potent vaccine candidate. Molecular docking of OmpK with TLR-5 was carried out by ClusPro2. The structural analysis produced a high-quality model that interacted well with TLR-5.

We stably expressed outer membrane protein K (OmpK) in *Nicotiana tabacum* via *Agrobacterium*-mediated transformation. *OmpK* gene was cloned using the Gateway® cloning. The Polymerase Chain Reaction (PCR) was done to confirm the proper integration of *OmpK* transgene in the transformed plants. The transgene copy number was calculated via qRT-PCR as two. The transgenic protein checked with Western blot showed monomeric form of OmpK. ELISA showed a maximum expression up to 0.38% of OmpK protein in the soluble fraction of total plant protein. Mice and fish were immunized with plant derived OmpK antigen, both showed significantly high

levels of anti-OmpK antibodies. The maximum antibody titer was recorded for subcutaneously vaccination with OmpK transgenic protein.

Edible plants can be used as alternative bio-factories to produce vaccine antigens. In this study, OmpK was also transiently expressed via *Agrobacterium*-mediated transformation in edible plants (*Spinacia oleracea* L. and *Lactuca sativa* L.). OmpK transgene expression in the infiltrated leaves of spinach and lettuce was confirmed through PCR which showed expression of OmpK in all leaves which was diminishing with time. OmpK protein was detected through Western blotting which showed a monomeric OmpK on the $2^{nd}$ to $5^{th}$ day in case of lettuce. Whereas in the case of spinach, the band was detected on the $2^{nd}$ to $4^{th}$ day, but no OmpK band was detected on the $5^{th}$ day. ELISA-based quantification of OmpK revealed maximum expression in lettuce (0.45%) and spinach (0.35%) on $3^{rd}$ day post agroinfiltration. The expression of OmpK in the edible plant could help in the development of an efficient oral subunit vaccine against vibriosis.

The present study is the first report of OmpK antigen expression in higher plants for the potential use as a vaccine in aquaculture against vibriosis, which could protect infection from multiple *Vibrio* species due to the conserved nature of OmpK antigen.

# *INTRODUCTION*

*Chapter 1*

Aquaculture encompasses all forms of aquatic organisms as well as aquatic plants in fresh, brackish, and marine ecosystems. Aquaculture is one of the largest, most diverse, intensive, and alternate food worldwide for humankind providing around fifty percent of all seafood for human consumption. To achieve planned results in aquaculture globally, it is crucial to employ innovative methods based on scientific, environmental, technical, and economic principles (Almeida *et al*., 2009; Araujo *et al*., 2022; Dawood *et al*., 2019; FAO, 2022; Natnan *et al*., 2021). Humans have long depended on fish protein as a vital food source. For an estimated three billion people worldwide, fish and other seafood species play a crucial role in providing essential nutrients and supporting the economy and social life. In short, fish alone accounts for approximately 20% of animal protein for three billion people (Steenson & Creedon, 2022).

In 2020, out of total fish capture of 90.5 million metric tons, aquaculture contributed 84.1 million metric tons. Freshwater aquaculture alone accounted for a significant portion, with 59.9 million metric tons (62.2%) of the world's total. In the same year, farmed fish reached 63.6 million metric tons, with 54.12 million metric tons originating from inland aquaculture and 9.14 million metric tons from marine and coastal aquaculture. Additionally, according to the Food and Agriculture Organization (FAO, 2020), freshwater fish followed by seaweed were the most significant organisms in terms of the global importance of aquaculture for food production. In 2020, carp and trout dominated aquaculture production, accounting for 150,000 tonnes of fish. Global fish consumption is anticipated to be contributed by aquaculture up to 57% by 2025 (Araujo *et al*., 2022; Arechavala-Lopez *et al*., 2022; FAO, 2022; Kaczan & Patil, 2020; Natnan *et al*., 2021; Shahbandeh, 2022).

Effective resource management plays a crucial role in ensuring global nutritional quality and food security, especially considering the rapidly increasing world population and the challenges posed by factors such as fish diseases, increased aquaculture production, stagnant catch fisheries production, climate change, and the competing demands for natural resources (Guillen *et al*., 2019). Fish diseases caused by microbes, even though the range of infecting pathogens may appear limited, are a global concern affecting the various type of fish, including freshwater, marine, feral, cultured, sport, and ornamental species. In aquaculture, fish are susceptible to illness due to routinely encountering bacterial diseases, which can significantly impact their

successful production. Furthermore, fish is highly vulnerable to rapid mortality in intense culture conditions due to bacterial disease (Preena *et al*., 2020; Trust, 1986).

### 1.1. Fish Diseases

Infectious diseases are those that are spread by microorganisms such as bacteria, viruses, fungi, or parasites. Under normal circumstances, the immune system of the host perfectly prevents the manifestation of disease. However, diseases can be caused by microorganisms either by interfering with the body's normal processes or stimulating the immune system, resulting in high fever, inflammation, and other defensive responses. Additionally, the disease will also occur if the host has a compromised immune system and the infectious agent overwhelms its immune system (Furuse, 2019; Yilmaz *et al*., 2022). Aquaculture continues to expand so that people can get healthy and nutritious protein. However, various factors, including infectious diseases, declining water quality, and environmental constraints, have made fish farming less profitable and less secure. The interaction between the pathogen, host, and environment plays a crucial role in the onsets of fish disease, making it a significant area of aquaculture research (Ji *et al*., 2020; Toranzo *et al*., 2005; Yilmaz *et al*., 2022).

Microbial attacks pose a constant threat to cultured fish. Waterborne fish diseases in aquaculture primarily involve infectious agents such as bacteria, fungi (to a lesser extent), helminths, oomycetes, protists, and viruses (Table 1.1). Bacterial diseases, in particular, represent significant challenges for the aquaculture industry resulting in significant losses to several fish farming systems (Alderman, 1996; Shao, 2001; Wahli *et al*., 2002). Microbes that contaminate fish with having bad implications on public health are classified into two main categories: indigenous microflora, which naturally occurs in the environment and includes species such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Photobacterium damselae*, *Vibrio anguillarum*, and other *Vibrio* spp., and non-indigenous microflora, which are introduced through environmental contamination from sources like domestic human waste and/or animal waste (e.g., Enterobacteriaceae such as *Escherichia coli* and *Salmonella* sp.) (Almeida *et al*., 2009; Fukuda *et al*., 1996; Iida *et al*., 1997; Kiyokuni *et al*., 1987; Muroga *et al*., 1986; Nakai *et al*., 1999; Nakai & Park, 2002; Toranzo *et al*., 2005).

In both natural and aquaculture systems, the two primary diseases infecting marine and brackish fish are vibriosis and photobacteriosis (Pasteurellosis) worldwide. Although

enteric bacteria in controlled fish farming die off quickly but still a significant number of these bacteria exist in the exteriors and the gut of fish; being hazardous to the well-being of consumers (Almeida *et al*., 2009; Toranzo *et al*., 1991). Without a systematic plan to combat pathogenic bacteria, viruses, or other microorganisms; aquaculture farming may not be able to meet the growing demands which may lead to overfishing of wild fish (Natnan *et al*., 2021).

**Table 1.1:** List of fish diseases, causative agents, and affected fish species.

| Diseases | Causative Agent | Affected Fish Species |
|---|---|---|
| **Bacterial Diseases** | | |
| **Vibriosis** | *Vibrio* species | Groupers, Barramundi, Seabass, Sole, and Seabreams |
| **Furunculosis** | *Aeromonas salmonicida* | Salmonid species, Trout, Charr, Grayling, Turbot, and Halibut |
| **Pasteurellosis** | *Photobacterium damselae* subsp. piscicida. | Seabass, Cobia, Yellowtail, and Seabream |
| **Viral Diseases** | | |
| **Viral nervous necrosis (VNN)** | *Piscine nodavirus* | Mostly grouper, *Anguilla* (Anguillidae), *Gadus morhua* (Gadidae), *Umbriana cirrose* (Sciaenidae), and *other species* |
| **Fish lymphocystis disease (FLD)** | Fish lymphocystis disease virus (FLDV) | *Epinephelus bruneus*, *E. malabaricus*, *E. chlorostigma*, and *E. fuscoguttatus* |
| **Parasitic Diseases** | | |
| **Amyloodinioniosis (Velvet disease)** | *Amyloodinium ocellatum* | *Epinephelus* spp. and *Cromileptes altivelis* |
| **Cryptocaryonosis/ White spot disease** | *Cryptocaryon irritans* | *Larimichthys crocea* |
| **References** | | |
| (Albert & Ransangan, 2013; Byadgi *et al*., 2019; Colorni & Diamant, 2014; de Ocenda *et al*., 2017; Devadason, 2018; H. T. Dong *et al*., 2017; Du *et al*., 2015; Haenen *et al*., 2014; Labella *et al*., 2006; Lokanathan *et al*., 2016; Low *et al*., 2016, 2017; Marana *et al*., 2017; Nagasawa & Cruz-Lacierda, 2004; Natnan *et al*., 2021; Ni *et al*., 2018; Nishi *et al*., 2016; Pham *et al*., 2020; Qiao *et al*., 2016; Volpe *et al*., 2020; C. M. Wen, 2016; Yingxue *et al*., 2006; Zorriehzahra, 2020; Zorrilla *et al*., 2003) | | |

## 1.2. Vibriosis

Vibriosis is an infection caused by bacteria belonging to the *Vibrionaceae* family. It is one of the leading causes of death of shrimps and marine/freshwater fish resulting in

severe economic loss to the aquaculture industry worldwide. It also poses a risk to human health. Most virulent *Vibrio* sp. is mostly a multi-host pathogen, while *Vibrio vulnificus* is a true zoonotic pathogen. Vibriosis exhibits a distinct seasonal distribution in regions with temperate climates. Among animal vibriosis, fish vibriosis has been the most extensively studied. *Vibrio* species are unique in that they have multiple entry points into their hosts. They infect fish either by colonizing the gills, anus, and/or intestine upon contact with pathogen-contaminated water or food. Outbreaks of vibriosis in farmed fish are also attributed to changes in the physicochemical characteristics of the water and overpopulation (Amaro *et al*., 2020; P. Kumar *et al*., 2015; Rasmussen *et al*., 2018; Yilmaz *et al*., 2022).

Vibriosis-related deaths among diverse species of fish raised in aqua-farms have been documented recently all over the world (H. T. Dong *et al*., 2017; Rameshkumar *et al*., 2017; S. R. Sharma *et al*., 2016; Z. M. Zhu *et al*., 2018). Extensive investigations into the transmission of vibriosis in aquaculture have significantly contributed to understanding the disease's etiology and progression. However, despite efforts, it is still not entirely apparent how vibriosis develops, partially due to the vast genetic and biological diversity of *Vibrio* species in aquatic environments, which makes it challenging to comprehend the diseases brought on by specific species. Furthermore, the dearth of research on fish infections caused by less often documented *Vibrio* species (aside from *Vibrio anguillarum*, *Vibrio harveyi, Vibrio ordalii*, and *Vibrio parahaemolyticus*) has hampered the development of effective therapeutic and protective strategies to combat vibriosis (N. Mohamad *et al*., 2019).

### 1.2.1. History of vibriosis

An infection was noticed in 1718, and it was later determined that it was brought on by a *Vibrio* species. It was identified as vibriosis much later. When *Bacillus anguillarum* caused epizootic diseases among migrating eels (*Anguilla vulgaris*), in1817, was the first confirmed case of infection caused by *Vibrio* species (Canestrini, 1893; Colwell & Grimes, 1984; Mancuso *et al*., 2015). In 1909, the bacterium was isolated from the European eels (*A. anguilla*) in Sweden during an epidemic outbreak and it was named *V. anguillarum*. In 1932, outbreaks of red disease caused significant mortalities among Danish water eels. Due to similarities between the pathogen and disease pathology, it was later hypothesized that the red disease outbreaks were caused by *V. anguillarum*. Subsequent outbreaks in wild fish, including saithe, and cod due to *Vibrio* species, were

documented in the following years (Bergman, 1909; Bruun & Heiberg, 1932; Samuelsen *et al*., 2006).

Vibriosis started to gain considerable attention when it began to threaten fish farming, particularly in Europe, Japan, North America, and the United States, leading to mortalities in the early 1970s. In Norwegian salmon farms in 1979, *V. salmonicida* caused a large-scale outbreak of Hitra disease. In later years, Atlantic salmon (*Salmo salar*) farming was severely impacted by winter ulcer disease due to *V. wodanis* (synonym *AliiVibrio wodanis*) and *V. viscosus* (synonym *Moritella viscosa*) in Iceland, Norway, and Scotland. Since 1963 in Asia, *Vibrio* infections have also impacted Japan's yellowtail (*Seriola quinqueradiata*) aquaculture farms. Vibriosis has also claimed losses in Japan's farmed coho salmon and seabream (Austin & Austin, 2016; Kashulin *et al*., 2017; Sindermann, 1984, 1990).

### 1.2.2. Taxonomy of vibriosis

The taxonomy of *Vibrio* spp. has undergone continuous revisions with many names proposed for *Vibrio* spp. infections, often by the scientists who first identified them. These names include cold pest, eye disease, red disease, red pest, and ulcer disease. Generally, the infection known as vibriosis is caused by several *Vibrio* species and those closely associated with genera *AliiVibrio* and *Photobacterium*. The advancement of species identification techniques over the years has led to significant changes in the taxonomic position of the *Vibrionaceae* family which now embraces 12 validly named genera. Through Multi-locus sequence analysis (MLSA), scientists have analyzed the sequences of different gene loci and divided more than 190 verified *Vibrio* spp. into nine genera: *AliiVibrio, Echinimonas*, *EnteroVibrio*, *Grimontia*, *Listonella*, *Photobacterium*, *SaliniVibrio*, *ThaumasioVibrio*, and *Vibrio* (Amin *et al*., 2017; Gomez-Gil *et al*., 2021; Noga, 2010; Sawabe *et al*., 2007, 2013; Yilmaz *et al*., 2022). Other genera in this family include *Catenococcus*, *Paraphotobacterium*, and *Veronia*. Some species are more commonly referred to as opportunistic pathogens, nevertheless regarded as pathogenic, rely on other risk factors to cause an epidemic and contribute to disease burdens in commercially important aquatic fish species (Amaro *et al*., 2020; Gomez-Gil *et al*., 2021; N. Mohamad *et al*., 2019; Parte *et al*., 2020).

### 1.2.3. Microbiology and habitat of *Vibrio* spp.

The *Vibrio* genus includes ubiquitous aquatic bacteria as well as crucial zoonotic pathogens like *Vibrio cholera*, *V. parahaemolyticus*, and *V. vulnificus*. In addition to these zoonotic species, some cause high mortalities in several cultured fish species include *V. anguillarum*, *V. harveyi*, *V. ordalii*, and *V. alginolyticus*. Several newly characterized species of *Vibrio* have also been identified, but their pathogenic and zoonotic roles are still unknown (Duman *et al*., 2023; N. Mohamad *et al*., 2019).

*Vibrio* spp. is comma-shaped or straight rod-like gram-negative mesophilic bacteria measuring approximately 1 µm in width and 2–3 µm in length. They possess one or more sheathed polar flagella and have two circular chromosomes (one large and one small). These bacteria are non-spore-forming facultative oxidase-positive bacteria. Most members of the *Vibrio* family are considered environmental microorganisms found in both fresh and saltwater (1-2% NaCl) environments. They play a vital role in maintaining the carbon and other nutrients cycles, creating a balanced microenvironment that benefits numerous aquatic creatures (Amaro *et al*., 2020; Gomez-Gil *et al*., 2021; N. Mohamad *et al*., 2019; Parte *et al*., 2020). *Vibrio* spp. typically ferments D-fructose, glycerol, and maltose, and they convert nitrate to nitrite. They are commonly found in the maritime ecosystem and are frequently associated with marine environmental particles without causing any adverse effects. *Vibrio* spp. can form biofilm in sediment or by clutching to colloids, trash, floating debris, marine snow, and host organisms. They have been isolated from different aquatic plants, the water column, and sediment as well as vertebrate and invertebrate creatures (A. Ali *et al*., 2020; E. Chase *et al*., 2015; Givens *et al*., 2014; Raszl *et al*., 2016; Soto, 2022).

These bacteria are temperature-dependent and renowned for adaptability, enabling them to survive in seawater under challenging circumstances. Although they have been reported in freshwater and lake habitats, many *Vibrio* spp. are halophiles (Sudheesh *et al*., 2012; Thompson *et al*., 2004; Vezzulli *et al*., 2015). Important species in the family *Vibrionaceae* that are distinguished to have devastating impacts on aquaculture fish include *V. alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. carchanae*, *V. cholera*, *V. damsela*, *V. harveyi*, *V. ordalii*, *V. owensii*, *V. parahaemolyticus*, *V. ponticus V. salmonicida*, and *V. vulnificus*. Additionally, *Vibrio mimicus*, non-halophilic, causes significant losses in freshwater-farmed fish (Andreoni & Magnani, 2014; Austin *et al*.,

2012; Bellos *et al*., 2015; Haenen *et al*., 2014; M. N. Kim & Bang, 2008; S. Liu *et al*., 2018; Sandlund *et al*., 2010).

### 1.2.4. *Vibrio anguillarum*: an important fish pathogen

*V. anguillarum*, also known as *Listonella anguillarum*, is a halophilic, gram-negative, comma-shaped, and non-spore-forming bacteria. Its mortality is facilitated by a single polar flagellum. *V. anguillarum*, the most common member of *Vibrionaceae*, was first identified in 1909 as the pathogenic agent for causing hemorrhagic septic infection in eels (*Anguilla anguilla*) and was initially named *Bacillus anguillarum*. The disease it caused was referred to as red eel pest. *V. anguillarum* is responsible for 'Classical Vibriosis' (Va-Vibriosis) and is known to infect a wide range of economically significant warm- and cold-water aquatic species. It thrives in estuarine and marine habitats and can grow across various salinity (1-2% NaCl) and temperatures (15˚C-17˚C). Among the *Vibrio*naceae family, *V. anguillarum* is most extensively studied species and gained significant attention as a model organism for studying the pathophysiology of vibriosis in fish (Amaro *et al*., 2020; L. T. Hickey *et al*., 2019; M. E. Hickey & Lee, 2018; Higuera *et al*., 2013; Ina-Salwany *et al*., 2019; Karami *et al*., 2020).

Among the 23 different serotypes, only the O1, O2 (O2α and O2β), and O3 serotypes of *V. anguillarum* are associated with fish mortality (Noga, 2010; Torres-Corral *et al*., 2021). In northern Europe, serotype O2a of *V. anguillarum* is the most found serotype in non-salmonid species. In 2008 in commercially valuable fish sp. saithe (*Pollachius virens*), a case of vibriosis was reported which was attributed to *V. anguillarum* serotype O2b. Serotype O1 is frequently isolated from *Dicentarchus labrax* (Europe), salmonid species (Norway), and *Sparus aurata* (Mediterranean). Investigations into intraspecific genetic diversity have revealed the presence of several clonal lineages of epidemiological significance among the most prevalent pathogenic serotypes (Dierckens *et al*., 2009; Hellberg *et al*., 2010; Toranzo *et al*., 2017).

*V. anguillarum* infection has been documented in over 50 freshwater and marine species, including Atlantic salmon (*Salmo salar*), *Gadus morhua* (Atlantic cod), *Oncorhynchus mykiss* (Rainbow trout), *Hippoglossus hippoglossus* (Atlantic halibut), *Cynoglossus semilaevis* (Tongue sole), *Psetta maxima* (Turbot), seabream, *Morone saxatilis* (Striped bass), sea bass, and *Plecoglossus altivelis* (ayu) (Higuera *et al*., 2013;

Nagai *et al*., 2008; Pedersen *et al*., 2008; Toranzo *et al*., 2005; Yilmaz *et al*., 2022; X. Zhang *et al*., 2015).

### 1.2.5 Transmission of vibriosis

Understanding the dynamics of pathogen transmission among vulnerable populations is crucial for effective management of infectious diseases. However, *Vibrio* spp. transmission mechanism remains unclear in aquaculture fish. Aquaculture farms can serve as reservoirs for pathogenic *Vibrio* strains, which can maintain their infectivity for extended periods. Farms with high density and long establishment along with sediment-filled environments are more likely to act as suitable reservoirs for vibriosis. Sediments rich in organic matter provide favorable conditions for bacterial biofilm formation. Predicting disease outbreaks requires well understanding that how pathogens transit from reservoirs or through the environment into susceptible hosts (E. Chase *et al*., 2015; Norfolk *et al*., 2023; Vezzulli *et al*., 2015).

*Vibrio* spp. have diverse ecological niches and engage in complex interspecies interactions. As a water-borne infection, *Vibrio* spp. naturally spread through the water medium. The horizontal spread of *Vibrio* spp. can occur through open wounds or by their release in the fecal material of carrier and/or infected fish. The water column may serve as a medium for *Vibrio* spp. to spread among neighboring fish. There is potential for vibriosis to spread from wild fish to farmed fish, as evidenced by plasmid profiles during epidemiological studies. While direct transfers are rare, horizontal transmission of cold-water vibriosis has been observed between wild-caught and farmed fish. Different serotypes of vibriosis may be found in reservoirs, prey, and wild fish. Wild aquatic birds increase the risk of *Vibrio* infections in both natural fish populations and can act as transmission vehicles and reservoirs for *Vibrio* spp. (Fernández-Delgado *et al*., 2016; Johansen *et al*., 2011; Norfolk *et al*., 2023).

*Vibrio* spp. present in the fish digestive tract is less affected by unfavorable environmental conditions compared to those in the marine environment. They can persist in asymptomatic fish that have previously experienced a vibriosis outbreak or in healthy fish as an integral part of its microbiome, potentially infecting other vulnerable fish. It is still unclear what is the exact function of different species of *Vibrio* that have been isolated from fish guts. Contaminated or infected sources such as fish eggs, food, rough fish, fish tools, young fish, hatcheries, breeding stock, and polluted upstream

farm water can introduce *Vibrio* spp. in culture fish (Kumara & Hettiarachchi, 2016; Lafferty *et al*., 2015; Noorian *et al*., 2023; Nurliyana *et al*., 2019; Sainz-Hernández & Maeda-Martínez, 2005).

## 1.3. Economic losses, mortalities, and other impacts due to vibriosis

### 1.3.1. Economic losses

Many countries around the world experience significant economic losses, estimated to be 10-15% of total production, due to bacterial fish diseases. Vibriosis is among the most common bacterial fish infections affecting commercially important farmed prawns, marine, and freshwater fish species worldwide. It has been associated with substantial economic losses due to mortalities in aquaculture, reaching up to over 9 billion dollars annually (Austin & Austin, 2016; N. Mohamad *et al*., 2019; Sanches-Fernandes *et al*., 2022; Toranzo *et al*., 2005; Yilmaz *et al*., 2022). However, accurate information and reliable data are still scarce on how much vibriosis economically impacted the cultured fish industry. *V. anguillarum* alone costs the fish industry up to 500 million US dollars per year. Vibriosis outbreaks in the prawn sector have been so severe that they significantly reduced prawn production. In 1978, vibriosis-related losses in farmed yellowtail in Japan were estimated at up to 4.4 million US dollars. It has also cost the Asian shrimp industry about 4 billion US dollars as a loss from 2009-2018. *V. anguillarum* is responsible for economic losses of about 250 million US dollars per year in *Seriola* spp. and *Ctenopharyngodon idella*. In Norway, *Vibrio* infections in salmon farming have resulted in losses exceeding 100 million US dollars, making it a significant issue (Abdel-Latif *et al*., 2022; Flegel, 2012; Leaño & Mohan, 2012; Maldonado-Miranda *et al*., 2022; Sindermann, 1984).

### 1.3.2. Mortalities

Fish mortalities contribute significantly to economic losses associated with vibriosis. In Asian fish culture, *V. anguillarum*-induced mortalities are currently causing substantial losses. Preventing and treating *V. anguillarum* infections remains a top priority in aquatic research (Hjerde *et al*., 2008; K. Xu *et al*., 2022). Mortality rates can exceed 50% during outbreaks in cultured farm fish, particularly in young ones (Vanamala *et al*., 2022). Before vaccination, *V. salmonicida* caused mortalities exceeding 80% in Norway's aquaculture industry and up to 90% losses in Atlantic salmon farming. *Vibrio* spp. infections in Asia often result in moderate mortality rates,

but cumulative mortality can still be significant. Outbreaks of *V. harveyi* in Vietnam barramundi fish farms reported fertility rates of up to 40% and in Malaysian seabass fish farms it caused high mortalities (Albert & Ransangan, 2013; H. T. Dong *et al.*, 2017; Hjerde *et al.*, 2008).

*V. harveyi* infections caused acute deaths in farmed groupers (*E. awoara*) in China and moderate deaths in farmed *Solea tauvina* in Spain. *V. harveyi* also contributed to deaths in cultured silvery black porgy and brown spotted groupers in Kuwait (Saeed, 1995; Zorrilla *et al.*, 2003). *V. alginolyticus* has been responsible for significant fish mortalities worldwide. In Taiwan, farmed juvenile cobia experienced a cumulative fatality rate of 30% due to *V. alginolyticus*. *V. ordalii* poses a regular problem for Chile's top producers of farmed salmon. Severe mortality rates (up to 30%) were documented in Spain for farmed *Argyrosous regius*, *European seabass*, and *gilthead seabream*, although reports were limited (N. Mohamad *et al.*, 2019).

### 1.3.3. Indirect impacts

Vibriosis can indirectly impact the fish industry by hindering the growth of cultured fish without causing significant mortality. Researchers have observed a connection between delayed growth in fishes such as grouper (*E. coloides*), and the manifestation of *Vibrio* spp. It is supposed that the slow growth of fish is a result of reduced appetite and stress caused by vibriosis (Cano-Gomez *et al.*, 2009; Sun *et al.*, 2009). This slow growth leads to decreased productivity, increased costs, and a longer time for fish to reach the desired market size. Moreover, *Vibrio* spp. infections often result in visible skin lesions on fish, reducing their market value (N. Mohamad *et al.*, 2019).

Fish diseases not only impact profitability and production but also affect workers in processing plants, feed mills, and fish farms, impacting their food security, income, and job stability. These consequences extend to local communities, with the Philippines experiencing a significant 75% reduction in household income due to vibriosis infections (Somga *et al.*, 2002). Additionally, *Vibrio* infections hinder international fish trade (Halwart *et al.*, 2007). Challenges in properly assessing economic losses arise due to data scarcity, inadequate record-keeping by farm operators, difficulty in identifying the causative agents of fish diseases, and unpredictable outbreaks of vibriosis. These factors contribute to a lack of awareness among fish farmers about the devastating losses associated with vibriosis (N. Mohamad *et al.*, 2019).

### 1.4. Control and prevention of vibriosis

#### 1.4.1. Biosecurity measures

To minimize the risk of pathogen infections, implementing biosecurity measures is essential. Unfortunately, many farms neglect this crucial step in disease prevention. Biosecurity should be put into practice to maintain disease-free aquaculture facilities. Biosecurity and water quality control have proven effective in combating vibriosis, a transmissible disease, in aquaculture facilities (Lafferty *et al*., 2015; Leandro, 2021; Yilmaz *et al*., 2022). These biosecurity practices will decrease the transmission of disease and eradicate infections when present.

- **Fingerlings' quality:** One crucial biosecurity practice is ensuring high-quality fingerlings. The fingerlings must come from healthy and disease-free sources. Adequate disease management is essential in hatching facilities where fish reproduction, hatching, and upbringing throughout their early life stages occur. Procedures like ultraviolet (UV) disinfection, filter systems, and steam disinfection, are efficient in eradicating and reducing *Vibrio* contamination from the water in these facilities (Elston *et al*., 2008; Kumara & Hettiarachchi, 2016; N. Mohamad *et al*., 2019; Sainz-Hernández & Maeda-Martínez, 2005). The use of *Vibrio* spp. free fresh or sea water can be an effective, preventative, and good alternative approach to limit the spread of disease in hatching facilities. Implementing quarantine measures and sourcing fingerlings from reputable suppliers further reduces the risk of exposure to pathogens in the established populations (Fouz *et al*., 2000; N. Mohamad *et al*., 2019; Sainz-Hernández & Maeda-Martínez, 2005).
- **Husbandry management:** Efficient farm management is essential for maintaining biosecurity. It involves effective control of clinically infected animals, breeding stocks, carriers, and the environment through prompt diagnosis and constant surveillance of fish farming conditions. Monitoring early indications of unusual activity, abnormal clinical symptoms, and vibriosis-related fatalities is vital. Swift removal of dead fish is crucial to stop the spreading of disease. Practicing excellent hygiene is necessary for controlling vibriosis. Proper cleaning, disinfection, and drying out processes for farm equipment are required to halt the pathogen's spread (N. Mohamad *et al*., 2019; Yildiz & Visick, 2009).
- **Stress management:** Stress has a substantial role in the vulnerability of fish to pathogens, including vibriosis. Chemical, biological, and physical stresses are widely

recognized as the primary contributors to the emergence of vibriosis. Mitigating stress in farmed fish is vital and can be achieved through recommended biological approaches such as water quality management, maintaining an optimal stocking density, employing suitable chemical utilization techniques, and supplementing food with vitamins and immunostimulants (Austin & Austin, 2016; P. Kumar *et al*., 2015). Aquaculture fishes and other species can adjust themselves to these natural oscillations to some extent, excessive stress can be detrimental. Therefore, for fish growth, productivity, and survival it is essential to maintain good water quality. Crucial factors like salinity, temperature, turbidity, nutrients (Nitrogen and phosphate) levels, and pH level should be monitored. These water characteristics also influence plant growth, thereby affecting the profitability of aquaculture. Additionally, factors such as dissolved oxygen, carbon dioxide, and ammonia act as stressors and impact the growth of aquaculture animals (Colt, 2006; N. Mohamad *et al*., 2019).

### 1.4.2. Antibiotics

Various antibiotics and antimicrobial drugs have been utilized for years to prevent vibriosis (Laganà *et al*., 2011; Yano *et al*., 2014). The majority of *Vibrio* spp. are often susceptible to antibiotics, which is the most employed method to treat bacterial infections in aquaculture. Acceptable antibiotics that have been used to treat vibriosis include flumequine, nitrofurans, oxolinic acid, oxytetracycline, quinolones, sarafloxacin, sulfonamides, sulfaisozole, sulphamonomethioxine, tetracycline, and trimethoprim (M. E. Hickey & Lee, 2018; Laganà *et al*., 2011; Rico *et al*., 2012; Yano *et al*., 2014). For example, oxytetracycline was administered to *V. alginolyticus*-infected farmed cobia for consecutive eight days, which completely reduced mortality. In another study done on Atlantic cod, a reduced mortality rate has been observed on oral administration of oxolinic acid and florfenicol against *V. anguillarum* (Rajan *et al*., 2001; Samuelsen & Bergh, 2004).

However, the frequent overuse of antibiotics to treat infections in aquatic species that have been cultured in aquafarms can have negative consequences. In most cases, it is a primary cause that antibiotic-resistant bacterial strains have emerged due to the development of antibiotic-resistant genes (ARGs). ARGs development has enabled many species of family *Vibrionaceae* to acquire resistance to antibiotics, which also pollutes the aquatic environment and has serious public health consequences for fish consumers (Adel *et al*., 2017; Cabello *et al*., 2013; Letchumanan *et al*., 2016; Loo *et*

*al*., 2020; B. M. Rao & Lalitha, 2015; Yilmaz *et al*., 2022). Many countries have reported an increasing number of antibiotic-resistant *Vibrio* strains in aquacultures, resistant to as many as ten different antibiotic classes. Reports of *V. anguillarum* resistant to actinonin and nitrofurans have also been documented. Ongoing discoveries of new resistance genes reveal the continuous emergence of antibiotic resistance in bacterial species. These genes are frequently transferred through plasmids or other extrachromosomal mobile genetic elements, facilitating the dissemination of antibiotic-resistant factors (Igbinosa, 2016; H. N. K. Nguyen *et al*., 2016; Scarano *et al*., 2014; Singh *et al*., 2022; Y. Xu *et al*., 2017; N. Yang *et al*., 2021; Z. M. Zhu *et al*., 2018). This poses challenges in the treatment of vibriosis. Moreover, the prevalence of antibiotic resistance in human infections has also increased due to the overuse of antibiotics in fish farms (Elmahdi *et al*., 2016; Hernández-Robles *et al*., 2016).

It is clear from the emergence of antibiotic resistance species that the development of new categories of antibiotics that can effectively combat drug-resistant bacteria is necessary, yet the discovery of such compounds poses a considerable challenge. As a result, there have been global calls for a reduction in antibiotic use. Therefore, vaccines have been extensively developed worldwide with a focus on the protection rather than treatment of vibriosis. There is clear need for potential, safe, alternative, non-antibiotic-based, and environmentally friendly techniques for the prevention and treatment of bacterial disease to avoid the negative repercussions of antibiotic use (Abdel-Latif *et al*., 2020; Abdel-Tawwab *et al*., 2020; Abdel-Tawwab & El-Araby, 2021; Ahmadifar *et al*., 2021; Dawood *et al*., 2021; Heuer *et al*., 2009; Ji *et al*., 2020; H. N. K. Nguyen *et al*., 2016; N. Yang *et al*., 2021).

### 1.4.3. Vaccines

Fish vaccination involves administering pathogenic antigens to induce protective immune responses. It is an effective immunoprophylactic method for fish survival against challenging pathogens. The ideal vaccine should be safe for fish, deliverers, and human consumers. It should provide lasting immunity against multiple pathogenic strains, be affordable, and maintain efficacy during production. Simplicity and suitability for various fish species are also important factors (Wali & Balkhi, 2016; Yilmaz *et al*., 2022). Active vaccines against vibriosis can mitigate antibiotic use in aquaculture. Progress has been made in developing vaccines for fish to immunize

against *Vibrio* spp. Various fish vaccine types exist, such as DNA, inactivated, live attenuated, live vector, and subunit vaccines (Ji *et al*., 2020; Yilmaz *et al*., 2022).

Conventional vaccines are produced from pathogenic microbes and their metabolites by modifying them through the processes such as inactivation, artificial attenuation, and detoxification. These include live attenuated and inactivated vaccines (Baxter, 2007). Virulence factors, such as adhesion factors, extracellular products, iron uptake systems, lipopolysaccharides, outer membrane proteins, and proteases, play a crucial role as vaccine antigens. These factors enable pathogens to enter the host, evade host defenses, and proliferate which leads to illness (Ruwandeepika *et al*., 2012; L. Li *et al*., 2019). OmpK, OmpV, OmpW, VPA0548, VP0887, VP1019, and VP2309 have been identified as potential antigens for *V. parahaemolyticus* vaccines, while Groel, OmpU, and VAA are candidate antigens for *V. anguillarum* vaccines (Mao *et al*., 2007; B. Peng *et al*., 2016; Xing *et al*., 2017). To combat *Vibrio* spp. infections the promising strategies are subunit, DNA, and live vector. Successful vaccination against various pathogenic *Vibrio* spp., including *V. anguillarum*, *V. alginolyticus*, and *V. harveyi* has been documented. Large-scale trials in Spain demonstrated 99% survival in seabass vaccinated against *V. anguillarum* serotype O1 (Haenen *et al*., 2014; Hu & Sun, 2011; Liang *et al*., 2010; Raida & Buchmann, 2008).

Diverse vaccine types have been developed for fish vaccination and each type has several advantages and disadvantages shown in Table 1.2. (Ina-Salwany *et al*., 2019). To combat important infections due to bacteria and viruses in fish currently thirty vaccines are commercially available. These include bacterial vaccines against *A. salmonicida*, *Arthrobacter*, *E. ictalurid*, *Flavobacterium columnare*, *V. anguillarum*, *V. ordalii*, and *Yersinia ruckeri* among others, for various fish species such as grouper and salmonids. Additionally, vaccines also exist against viral diseases such as hemorrhage disease, infectious salmon anemia (ISA), infectious pancreatic necrosis (IPN), koi herpes virus disease (KHVD), meningoencephalitis and septicemia, and viral nervous necrosis (VNN) targeting species like carps, salmonids, seabass, and tilapia (H. Su *et al*., 2021).

#### 1.4.3.1. Inactivated vaccines

Inactivated vaccines use killed bacteria or viruses that have lost their pathogenicity but retained their antigenicity (Tlaxca *et al*., 2015). They are safe, easily stored, and free

from contamination risks. These vaccines eliminate and neutralize pathogenic microorganisms and their toxic metabolites by inducing a humoral immune response that generates specific antibodies against them (Baxter, 2007). Inactivated oral vaccines have been developed against *Vibrio* spp. For example, *Epinephelus coioides* have been protected from infections of *V. harveyi* via a formalin-inactivated vaccine (Duff, 1942; H. T. Nguyen *et al*., 2017). Similarly, a formalin-inactivated whole-cell vaccine effectively prevented diverse *Vibrio vulnificus* infections in tilapia. Vaccinated tilapia showed 88% relative percent survival (RPS). Inactivated vaccines also protected cultured seabass, seabream, and turbot against vibriosis (Gudding & Van Muiswinkel, 2013; Shoemaker *et al*., 2011).

Inactivated vaccines lack replicating antigens, unlike live attenuated vaccines. This necessitates multiple vaccinations. Other drawbacks include such as it has a single route, a high dose is required, and the period of immunization is too short. They primarily stimulate humoral immunity, lacking mucosal immunity. These vaccines often need adjuvants to boost or enhance their immunogenicity (Baxter, 2007; O'Hagan, 1998). Mineral oil adjuvants, commonly used, have serious side effects. These limitations have spurred the development of novel adjuvants, including aluminum-coated, lipopeptides, chitosan oligosaccharide, β-glucans, cytokines, nano/microparticles, saponins, toll-like receptor (TLR) 3 agonist polyinosinic polycytidylic acid (Poly I: C), TLR 5 agonist flagellin, and TLR 9 agonist CpG, (Gjessing *et al*., 2012; Kawakami *et al*., 1998; Tafalla *et al*., 2013).

#### 1.4.3.2. Live attenuated vaccines

Various treatments are used to reduce pathogen toxicity in live attenuated vaccines while maintaining immunogenicity. These vaccines activate the immune response for long-term protection without causing disease upon inoculation (Spreng *et al*., 2006). They replicate in the host, providing strong immunogenicity like natural infections. However, there is a potential danger for individuals with poor immunity and the restoration of virulence through mutation (Baxter, 2007). Live attenuated vaccines for fish can be administered through immersion immunization and intraperitoneal injection (Hu *et al*., 2012; Pang *et al*., 2018). The duration of immersion immunization significantly affects its immune effect, and reducing vaccine concentration and extending immunization time can enhance its effectiveness (Ji *et al*., 2020; J. D. Moore *et al*., 1998). Intraperitoneal injection primarily triggers an innate immune response and

is not suitable for immunizing juvenile fish, as their immature immune systems offer limited protection compared to broodstock (Pérez-Sánchez *et al*., 2018).

**Table 1.2:** Advantages and disadvantages of different types of vaccines.

| Advantages | Disadvantages |
|---|---|
| **Live vaccines** | |
| Attenuated vaccine | Laborious administration |
| Effectively stimulates immune system | High Cost |
| Immunity duration (6-12 months) | Inconvenient storage |
| *In vivo* reproduction of the pathogen | Poor safety |
| A low dosage is required | Short shelf life |
| **Inactivated vaccines** | |
| Inactivated vaccine | Laborious administration and high cost |
| Short development cycle | Pathogens cannot reproduce |
| Safe to use | Require multiple dosages |
| Easy to preserve | Short-term protection duration |
| | Appropriate adjuvant is needed |
| **Live vector vaccine** | |
| Low production cost | |
| Multiple vaccine possibles | Long-term immunity |
| Induce immunization *in vivo* | Harmful bacteria can become virulent |
| Low risk of virulence | |
| **DNA Vaccine** | |
| Safe, cost-effective, and well-tolerated | |
| Less need for cold chain delivery | Lower immunogenicity |
| Highly adaptable to a new pathogen | Difficult administration route |
| Auto-immune response | Risk of genomic integration |
| Native antigen expression | |
| **Subunit Vaccine** | |
| Promising recombinant vaccine | Affected by the expression system |
| Excellent safety and easy control | Uncertain immune responses |
| High stability and high purity | The tissue expression level is indefinite |
| Cost-effective and good immunogenicity | Short protection duration |
| Convenient for oral administration | Short protection duration |
| **References** | |
| (Du *et al*., 2022; K. H. Khan, 2013; J. Lee *et al*., 2018; Y.-D. Li *et al*., 2020; H. Su *et al*., 2021) | |

Specific genes are modified in wild-type strains to create attenuated vaccines. These genes can be divided into two types: virulence factors that manipulate the host immune system and cause cell death, and regulatory genes essential for strain growth. In a recent study, a mutant strain, *V. alginolyticus* hopPmaJ, has been developed by utilizing an overlap extension PCR. This mutant strain exhibited a 2600-fold reduction in virulence and stimulated multiple immune responses in *E. coioides* (Pang *et al*., 2018). Researchers developed *V. alginolyticus* mutants (ΔacfA, ΔsodB, ΔclpP) and *Vibrio anguillarum* auxotrophic strains (Δalr1 Δalr2) as live attenuated vaccines (Y. Chen *et al*., 2020; Y. Chen, Cai, *et al*., 2019 a; Y. Chen, Wu, *et al*., 2019 b; Choi *et al*., 2016). Antibiotic mutagenesis selects attenuated variants, like rifampicin-induced *V. harveyi* strain T4DM. These vaccines have shown immune-protective effects against *V. alginolyticus* and *V. harveyi* via immersion or injections (Baxter, 2007; Hu *et al*., 2012).

Genetically engineered live *V. anguillarum* vaccine (strain MVAV6203) approved in China in 2019 protects multiple fish species such as turbot, tiger puff, oliver flounder, and zebrafish with improved biosafety. Ensuring sufficient attenuation and minimizing strain persistence is crucial. Chu *et al*. established an iron-limiting signal-driven *in vivo* bacterial lysis system for safe *V. anguillarum* vaccination (RPS 89.3% in zebrafish). Given limited efficacy, new *Vibrio* spp. vaccines and antigens are under study (Chu *et al*., 2015; K. Xu *et al*., 2022). New vaccines and antigens are being studied to address the limited efficacy of traditional/conventional vaccines against large-scale *Vibrio* spp. infections (Ji *et al*., 2020).

#### 1.4.3.3. DNA vaccines

In 1990 scientists found that when the recombinant exogenous plasmid is injected into the muscle of a mouse it resulted in stable expression of the luciferase protein for an extended period (Wolff *et al*., 1990). This discovery led to the development of DNA immunological technology, where a recombinant vector harboring a specific protein or antigen is injected into a host directly. The vector is taken up by host cells and leads to the expression of the antigen protein, triggering immune responses (Ji *et al*., 2020). DNA vaccines have the advantage of acting as an adjuvant itself and can avoid misfolding and incomplete glycosylation. Such properties make DNA vaccines cost-effective (Dadar *et al*., 2017). DNA vaccines have been extensively studied for preventing and controlling vibriosis caused by *Vibrio* spp., offering a more efficient alternative to conventional vaccinations (P. Huang *et al*., 2019; S. R. Kumar *et al*.,

2008; Liang *et al*., 2011; R. Liu *et al*., 2011). Recombinant plasmids targeting genes like Hsp33, mutated EmpA, OmpK, OmpU, and Sia10 have been developed for *V. anguillarum* DNA vaccines, inducing immune responses against the infection (Sun *et al*., 2012; H. Xu *et al*., 2019a, 2019b). Bivalent DNA vaccines and the use of adjuvants have been explored to enhance their effectiveness (Hu & Sun, 2011; P. Huang *et al*., 2019).

However, intramuscular injection, the current method of administration of DNA vaccines, is impractical for large-scale field applications (J. Cao *et al*., 2019). To overcome this limitation, the development of oral vaccines is necessary, particularly for intestinal pathogens like *V. mimicus* (Rivas-Aravena *et al*., 2013). Bacterial ghosts and nano/microparticles, such as PLGA polymers and chitosan nanoparticles, have been investigated as delivery systems for oral DNA vaccines, improving their efficacy in preventing infections. These advancements contribute to the ongoing development of oral DNA vaccines (J. Cao *et al*., 2019; S. R. Kumar *et al*., 2008; Tafalla *et al*., 2013).

#### 1.4.3.4. Live vector vaccines

When a vaccine is created by inserting an immunogenic antigen-encoding gene into attenuated viruses or bacteria (live vectors) using different molecular biology techniques is referred to as a live vector vaccine. This results in the expression of antigens in the host, triggering an immune response for protection. Live vector vaccines have the accuracy of subunit vaccines and immunogenicity of live attenuated vaccines against pathogenic bacteria which makes them valuable for vaccine development. Bacteria serve as live carriers to activate the host's immune system, but virulent pathogens like *Salmonella* and *Listeria monocytogenes* (Lm) require attenuation before use (Ji *et al*., 2020; Maciag *et al*., 2009; Toussaint *et al*., 2013; Q. Wang *et al*., 2009). Early attempts at attenuation using chemical mutagenesis were limited and prone to virulence rebound. Genetic engineering techniques have enabled the targeted deletion of virulent genes in bacteria. Ding *et al*. created a live vector vaccine by inserting a gene encoding an outer membrane protein K (OmpK) of *V. parahaemolyticus* in an attenuated Lm strain (EGDe-ΔactA/inlB, Lmdd). This vaccine offered partial protection against *Vibrio* species in zebrafish (Ding *et al*., 2017, 2019; Ji *et al*., 2020).

#### 1.4.3.5. Subunit vaccines

Subunit vaccines consist of specific protein structures from bacteria, focusing on immunogenic non-toxic epitopes to eliminate adverse reactions caused by unrelated epitopes. Subunit vaccines can be directly purified from the desired pathogen or genetically engineered by transferring antigen-encoding genes to another organism. Both eukaryotic and prokaryotic expression systems are commonly used to produce *Vibrio* spp. vaccines based on immunogenic proteins (Ji *et al*., 2020).

Hamod *et al*. (2012) expressed in *Escherichia coli* the OmpK gene from *V. anguillarum* as a subunit vaccine with RPS 67.8% in carps. Researchers by combining different antigens have also developed multivalent subunit vaccines. Such as bivalent subunit vaccines were created by combining FlaC protein with other antigens, resulting in increased RPS values of up to 78.95% in flounders (Pang *et al*., 2016). Adjuvants, such as CpG-ODN 1668, have also been used to enhance the protective effect. For example, flagellin-A subunit vaccine survival rate is increased in vaccinated *Oreochromis niloticus* challenged with *V. anguillarum* when introduced in combination with adjuvant CpG-ODN 1668 (H. C. Kwon & Kang, 2016; Tafalla *et al*., 2013).

Identification of immunogenic antigens is a crucial step for subunit vaccine development. Reverse vaccinology and bioinformatics tools that rely on genomic data are used to analyze candidate antigen genes for antigenicity, virulence prediction, and subcellular localization. Techniques like biochip technology, signature mutagenesis, proteomics, and *in vivo* expression systems aid in discovering membrane protein antigens and/or invasion/virulence-associated antigens (D'Mello *et al*., 2019). For instance, Lun *et al*. analyzed the gene LamB in different strains of *Vibrio* via sequence alignment, phylogenetic tree construction, and epitope prediction. The study revealed that vaccine LamB can immunize *Danio rerio* against multiple *Vibrio* species (Ji *et al*., 2020; Lun *et al*., 2014).

Commercial vaccines like AlphaJect 2000™, Alpha Marine™, Aqua-Vac™, Micro ViB, Norvax-*Vibrio*, *Vibrio*-Pasteurella, and Aquavac *Vibrio* Oral® have shown effectiveness against various *Vibrio* infections. They have been widely used for around 15 years as effective preventive measures against vibriosis in aquaculture (Haenen *et al*., 2014; Mikkelsen *et al*., 2007; Spinos *et al*., 2017; K. Xu *et al*., 2022). However, vibriosis outbreaks still occur frequently in vaccinated fish due to the adaptability of

*Vibrio* spp. Yet, implementing successful vaccines often proves to be expensive and impractical, particularly in small and medium-sized operations. Challenges include the difficulty of vaccine administration, the need for a skilled labor force, and high costs. Therefore, developing a cost-effective, feed-based vaccination with cross-protection and easy application for farmers should be a top priority in the future (Karami *et al*., 2020; Yilmaz *et al*., 2022; Zamri-Saad *et al*., 2014).

### 1.5. Plant biopharming

Plants have long served as a valuable source of medicines, and many drugs are currently derived from them. They are also manipulated to increase food production and to produce different compounds. Plant breeding techniques were the primary means of crop improvement until the 19th century. However, advancements in genetic modification techniques for plants, which emerged through tissue culture and recombinant DNA technology, allow precise genetic manipulation (Chawla, 2011; Chemat *et al*., 2019). In developing countries, infectious diseases contribute to a high mortality rate, with vaccines playing a vital role in disease prevention. Vaccines have been used for over 200 years to control diseases (Charania *et al*., 2019; B. V. Kumar *et al*., 2013; Tiwari *et al*., 2009). Vaccines stimulate the production of antibodies, providing immune protection. However, the unavailability of vaccines for many diseases remains a challenge. Factors contributing to this include the high production costs, the inability to culture certain pathogens in exogenous media, and the need for proper storage, and distribution to maintain vaccine efficacy (Alpaugh & Cicchetti, 2019; Glick & Patten, 2022). Expensive fermenter-based production systems, costly media, and purification processes further contribute to the high costs of vaccines. Considering that the burden of disease primarily lies in developing countries, the cost-effectiveness of vaccine production is crucial for the development of next-generation vaccines (Burnett & Burnett, 2020; Lössl & Waheed, 2011).

#### 1.5.1. Plant expression platform

The synthesis of vaccine antigens in plants involves transferring disease-related genomes into plant host tissues. This can be achieved through stable transgenic plants or transient expression. Transgenic plants are created by introducing designed plant vectors via *A. tumefaciens* or using biolistic techniques, leading to the incorporation of foreign genetic material (Kurup & Thomas, 2020). Plant-based vaccine production

began in 1989 when immunoglobulin molecules were first expressed in transgenic plants. Edible vaccines gained interest due to their potential cost savings and ability to enhance mucosal immunity (Canto, 2016). Initially, various edible plant species were explored including carrots, bananas, lettuce, maize, potato, rice, spinach, and tomato. Chloroplast transformation has also been used for vaccine production. Previous attempts focused on vaccines against anthrax, cholera, Lyme disease, plague, rotavirus, and tetanus. Although transgenic plant production is time-consuming and may result in decreased yields (Balke & Zeltins, 2019; Kumari *et al*., 2023; Yamamoto *et al*., 2018). Producing recombinant plant vaccines with high expression levels, suitable for long-term storage and oral administration, is a major challenge (M.-Y. Kim *et al*., 2015). Advances in plant genetic manipulation have expanded the options beyond replication, including high protein content, and acceptable edible plants. Various plant platforms have been explored, each with advantages and limitations (Kumari *et al*., 2023).

- **Vegetables:** Certain vegetables, including potatoes, tomatoes, and carrots, are suitable for producing plant-derived vaccines due to their appealing nature, lack of toxins, high nutrient content, and freshness. Carrots have shown promising results with high levels of protein expression. Edible carrots offer the advantage of preserving the structural integrity of medicinal proteins without the need for preparation (Muller *et al*., 2003). Other genetically modified vegetable systems like lettuce, celery cabbage, and cauliflower are also being explored, although low transcription levels pose challenges in these plant gene expression systems (Kumari *et al*., 2023).
- **Fruits:** Bananas (*Musa acuminate*) have been early participants in plant transgenic programs and the activator MaExp1 has shown promise in expressing foreign proteins including vaccines in ripening banana fruit. Papaya (*Carica papaya*), a widely grown tropical and semi-tropical fruit, has also been genetically modified using bio-ballistics to produce vaccines. A synthetic vaccine called SPvac has been expressed in papaya, showing potential for large-scale vaccine production and exploring plant-based systemic and oral immunization in the future (Carter III & Langridge, 2002; Kumari *et al*., 2023).
- **Crops:** Certain spermatophytes (seed plants) are well-suited for producing oral vaccines due to their abundant soluble proteins and ease of storage. Alfalfa (*Medicago sativa*), a perennial crop with high regeneration ability, can rapidly generate large populations of clonal plants, making it suitable for long-term production. Its leaves

serve as an ideal bioreactor for recombinant protein production due to their high protein content and low levels of secondary metabolites (Stoger *et al*., 2005). Maize (*Zea mays*) and rice (*Oryza sativa* L.) have also been explored as commercial platforms for manufacturing medicinal and scientific proteins, leveraging their endosperms' rich soluble protein content to increase antigen concentration and reduce oral dosages (Hood *et al*., 2002; Kumari *et al*., 2023).

- **Algae and other halobios:** Researchers are investigating the potential of transgenic microalgae as green cell factories to produce beneficial phytochemicals and foreign proteins for medicinal use. Several species, including *Amphidinium carterae*, *Chlamydomonas reiihardtii*, *Cylindrotheca fusifornzis*, *Phaeodactylum triornutum*, and *Symbiodinium microadriatum* have been genetically modified. Algae-based fermentation for oral vaccines offers advantages over traditional methods, addressing challenges such as low expression, toxicity, lack of fresh edibles, and long growth times (Kumari *et al*., 2023; Mayfield & Franklin, 2005).

### 1.5.2. Plant transformation methods for subunit vaccine production

Molecular farming involves utilizing plants as production systems to generate recombinant proteins for specific purposes, rather than focusing on enhancing plant traits. This technique offers an economically feasible alternative to conventional production methods like large-scale bioreactors for microbial or mammalian cell cultivation (Bock, 2015; Buyel *et al*., 2017; Castells-Graells & Lomonossoff, 2021; Clarke *et al*., 2013; Dobrica *et al*., 2017). Three plant biotechnology platforms have been used to express monoclonal antibodies, antigens for vaccines, and other biopharmaceuticals: transient expression, stable nuclear expression, and stable plastid/chloroplast expression (Clarke *et al*., 2013; Daniell *et al*., 2009; Lössl & Waheed, 2011; Maliga & Bock, 2011; Streatfield, 2007). Each platform has its own merits and demerits, and the selection of the platform depends on the desired production requirements. This field has been explored in various studies, highlighting its potential for commercial-scale production of valuable proteins in plants (H. Su *et al*., 2021).

- **Stable nuclear transformation:** Plant cell nuclear transformation, pioneered around forty years ago, has primarily relied on the transfection of *Agrobacterium tumefaciens* (Figure 1.1) This technique involves the insertion of the DNA, for protein expression, into the binary vector as a transcriptional unit (promoter-gene-terminator). The plasmid, carrying the antigen's transcriptional unit, is then introduced into *A.*

*tumefaciens*, often through electroporation. Either organogenesis or embryogenesis process is employed for the regeneration of transgenic plants from nodes or leaf fragments after infecting them with *Agrobacterium*. Confirmation of successful genetic transformation in regenerated transgenic plants can be done by detecting the transgenes or proteins through different molecular biology techniques (Dandekar & Fisk, 2004; Monreal-Escalante *et al*., 2022). Recombinant binary vector harbors a fragment positioned between the T borders that incorporates into nuclear genetic material after infection during *Agrobacterium*-mediated nuclear transformation (Tzfira *et al*., 2004; Tzfira & Citovsky, 2006). This fragment includes a gene conferring antibiotic resistance for the selection of transformed plant cells. These transformed cells are then regenerated to fully grown plants on culture media supplemented with hormones, regulators, and antibiotics. In transgenic plants, the expression of genes of interest is either under the control of inducible or constitutive promoters. Inducible promoters are responsive to physical factors or substances such as light, temperature, alcohol, sodium chloride, or ethanol (Borghi, 2010; Corrado & Karali, 2009; I. Moore *et al*., 2006). Additionally, the gene copy number and protein yield can be enhanced by the incorporation of viral genetic elements into recombinant binary vectors (Arevalo-Villalobos *et al*., 2020; Dugdale *et al*., 2013). For the product of the vaccine, *Nicotiana tabacum* (tobacco) initially served as the primary model plant but with advancements in research, other plants including lettuce and carrots have emerged as promising candidates for oral vaccine formulation (Lai *et al*., 2012; Monreal-Escalante *et al*., 2016, 2022; van Eerde *et al*., 2019).

- **Transient nuclear transformation**: The transient nuclear expression approach is utilized when transformed plant cells need to be maintained for a short period without the need for antibiotic selection. In this method, high production of recombinant protein through messenger RNA synthesis is achieved by the introduction of multiple gene copies into the plant's nuclear genetic material (Musiychuk *et al*., 2007; Yusibov & Rabindran, 2008). Various strategies such as viral vectors or *Agrobacterium tumefaciens* carrying the antigen gene can be employed to achieve transient transformation and infect many plant cells (Yusibov *et al*., 2013). Viral vectors, including the *Tobacco mosaic* virus (TMV) and RNA-based vectors, are commonly used for transient expression with a diverse range of viral vectors available for transient expression (T. H. Chen *et al*., 2017; Gleba *et al*., 2005; Marillonnet *et al*., 2005). Plant *Gemini* viruses, leveraging the rolling circle replication mechanism, have been

extensively utilized for producing biopharmaceutical proteins (Q. Chen *et al*., 2011; Regnard *et al*., 2010; Rybicki & Martin, 2011). The effectiveness of viral vectors in producing high levels of antigen production relies on their ability to replicate in the plant cell by evading their immune system(Monreal-Escalante *et al*., 2022).

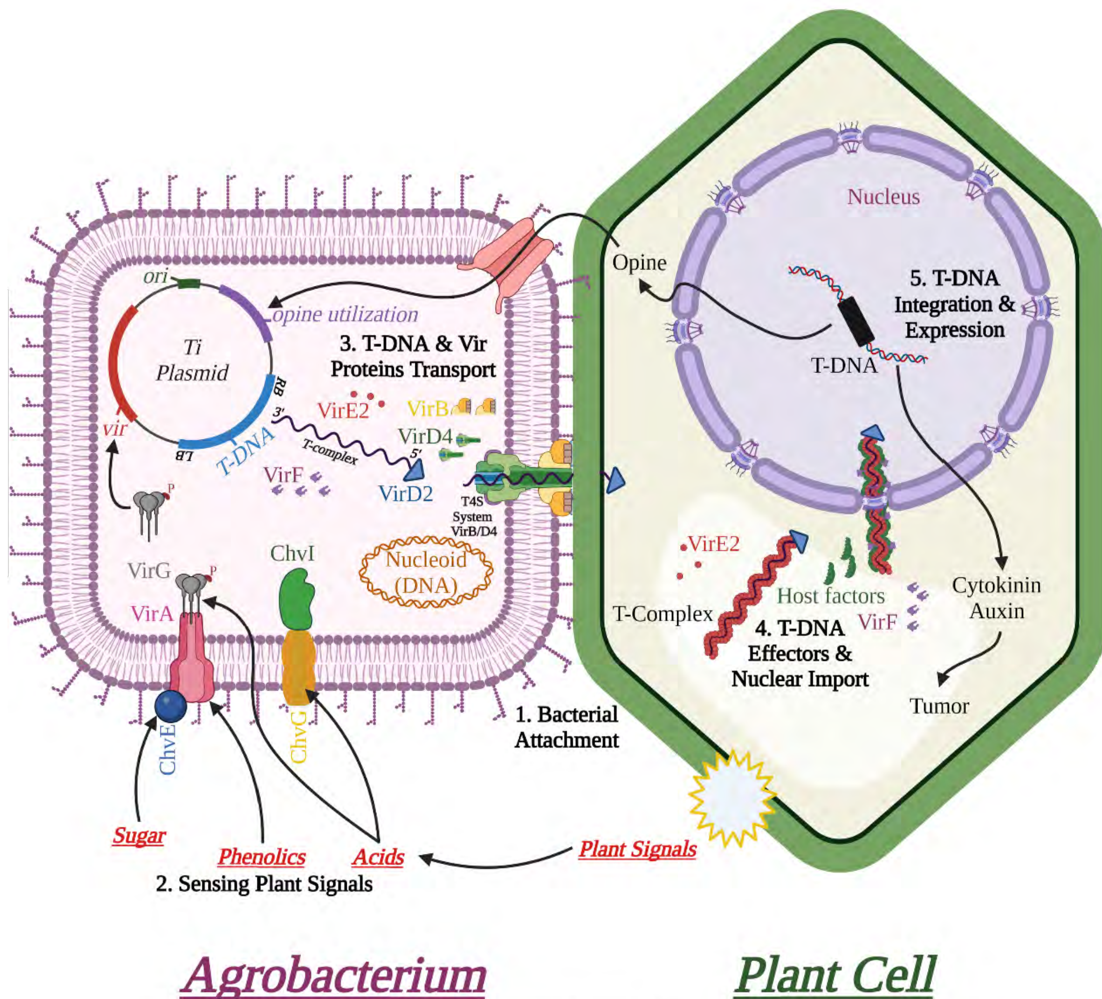



**Figure 1.1:** The integration mechanism of *Agrobacterium*'s T-DNA into the plant genome (Modified from H.-H. Hwang *et al*., 2017).

- **Chloroplast transformation:** This method marked the initial genetic modification of green cells primarily aimed at creating antibiotic-resistant cells. Biolistic procedures involving the delivery of genetic material-coated microparticles into target cells were initially employed to achieve chloroplast transformation. Within the cells, DNA fragments through the DNA recombination mechanism were integrated into the genome of the chloroplast. Through the same recombination mechanism transformation of other plastid organelles (chromoplasts) has also been achieved in tubers and fruits. Chloroplasts offer advantages for antigen production, including higher recombinant

protein yields due to the increased copy number in the genome of chloroplast in plants. However, chloroplasts lack the machinery required for post-translational modification, a limitation, which is required for the production of proteins that need post-translational modification for the development of vaccines (Clarke *et al.*, 2013; Monreal-Escalante *et al.*, 2022; Ruf *et al.*, 2001; Saba *et al.*, 2019; Verma & Daniell, 2007; Waheed *et al.*, 2015). The general process of plant-based vaccine production is shown in Figure 1.2.

### 1.5.3. Plant-based vaccines

Plant-based vaccines introduce pathogen antigens into selected plants for recombinant vaccine production. Over 200 proteins have been successfully expressed in plants, indicating a bright future for this approach. Hiatt and colleagues pioneered plant-based vaccine production in 1989, while the National Institute of Allergic and Infectious Diseases (NIAID) confirmed their immunogenicity in 1998. The first plant-based vaccine for the Newcastle disease virus (NDV) was approved by the United States Department of Agriculture (USDA) after eight years of development (Hiatt *et al.*, 1989; Pollard & Bijker, 2021). Dr. Arntzen and his team used transgenic plants to produce subunit vaccines, overcoming traditional manufacturing limitations. They created subunit vaccines in tobacco plants for *Streptococcus* mutants and explored hepatitis B and heat-resistant toxin B subunits in potatoes (Saxena & Rawat, 2014). Edible vaccines using plants as bioreactors demonstrated considerable immunogenicity and cost-effectiveness. Genetically modified plants offer a revolutionary solution, minimizing production and storage costs while reducing risks associated with pathogens and contaminants (Márquez-Escobar *et al.*, 2017; Sartaj Sohrab *et al.*, 2017; Sohrab, 2020).

A new oral vibriosis vaccine has been developed for use in Asian sea bass (*Lates calcarifer*), showing effectiveness in fish by stimulating both innate and adaptive immunological reactions (A. Mohamad *et al.*, 2021; H. Mondal & Thomas, 2022). Plants serve as smart bioreactors for the large-scale synthesis of vaccines, antibodies, and other bioactive compounds. Plant-based vaccines provide needle-free and convenient delivery for fishes particularly. Various plants like tobacco, potato, tomato, maize, and rice act as bioreactors (L. Joshi & Lopez, 2005; Saxena & Rawat, 2014). Transgenic plants are used for manufacturing bacterial, viral, parasite, and immune contraceptive vaccines. Several plant-based vaccines are in clinical trials, particularly targeting fatal diseases caused by viruses and bacteria. *Nicotiana* plants are commonly

employed as bioreactors (Davoodi-Semiromi *et al*., 2010). Two licensed products include a plant-made scFV mAB for recombinant HBV vaccine production in Cuba and a USDA-approved vaccine for poultry against Newcastle disease virus (NDV). However, the US Food and Drug Administration (FDA) hasn't approved any plant-based vaccines due to their classification as genetically engineered crops (Kumari *et al*., 2023). Recently, there has been a wide range of plant-made vaccines, which can be categorized into different types.

- **Virus-based nanoparticle (VNPs):** Plant-made vaccines have shown great potential for the synthesis of virus-like particles (VLPs) particularly in recent years. VLPs are structurally like wild-type counterparts but lack infectious nucleic acid. They can effectively stimulate the human immune system and are easily produced in large quantities in plants. Different viruses of plants, such as *Cowpea mosaic* virus (CPMV), potato virus X (PVX), and *Tobacco mosaic* virus (TMV) have been utilized for VLP production. Some VLPs, like those for *Human papillomavirus* (HPV), are easier to create compared to enveloped viruses like influenza (Nooraei *et al*., 2021; Vahdat *et al*., 2021). Plant-based VLPs can also be modified to display immunogenic epitopes on their surface, further enhancing their effectiveness as vaccine carriers. Another breakthrough is the development of pseudovirions (PSVs), which contain heterologous RNA or DNA encoding desired antigens. These PSVs have shown potential in delivering antigens into target cells. Tandem Core Technology, involving the replication of Hepatitis B core antigen (HBcAg) dimers, has been used to overcome challenges related to antigen crowding. Overall, plant-based vaccines, including VLPs and PSVs, offer a safe and scalable approach to vaccine production with great potential for future applications (Peyret *et al*., 2015; Vahdat *et al*., 2021; Y. Zhou *et al*., 2015).
- **Recombinant Immune Complexes (RICs):** Antigens and antibodies interaction results in the formation of immune complexes (ICs). They play a crucial role in activating both adaptive and innate immunological responses, including antibody-dependent cell-mediated cytotoxicity, $CD8^{+}$ and $CD4^{+}$ cell activation, complement-dependent cytotoxicity, and cross-presentation. Recombinant ICs (RICs) are a modified form of ICs that involve fusing the antigen to the C-terminus of a monoclonal antibody (mAb). This modification allows the RIC to effectively engage with the immune system (Kumari *et al*., 2023; X.-Y. Wang *et al*., 2019). Researchers have successfully designed hybrid and enhanced immunogenic RICs to target multiple viruses like dengue, Ebola,

and Zika virus. These advancements in RIC production demonstrate the plant-based system's potential in generating effective immune responses against viral infections (Diamos *et al*., 2020; M.-Y. Kim *et al*., 2015; Phoolcharoen *et al*., 2011).

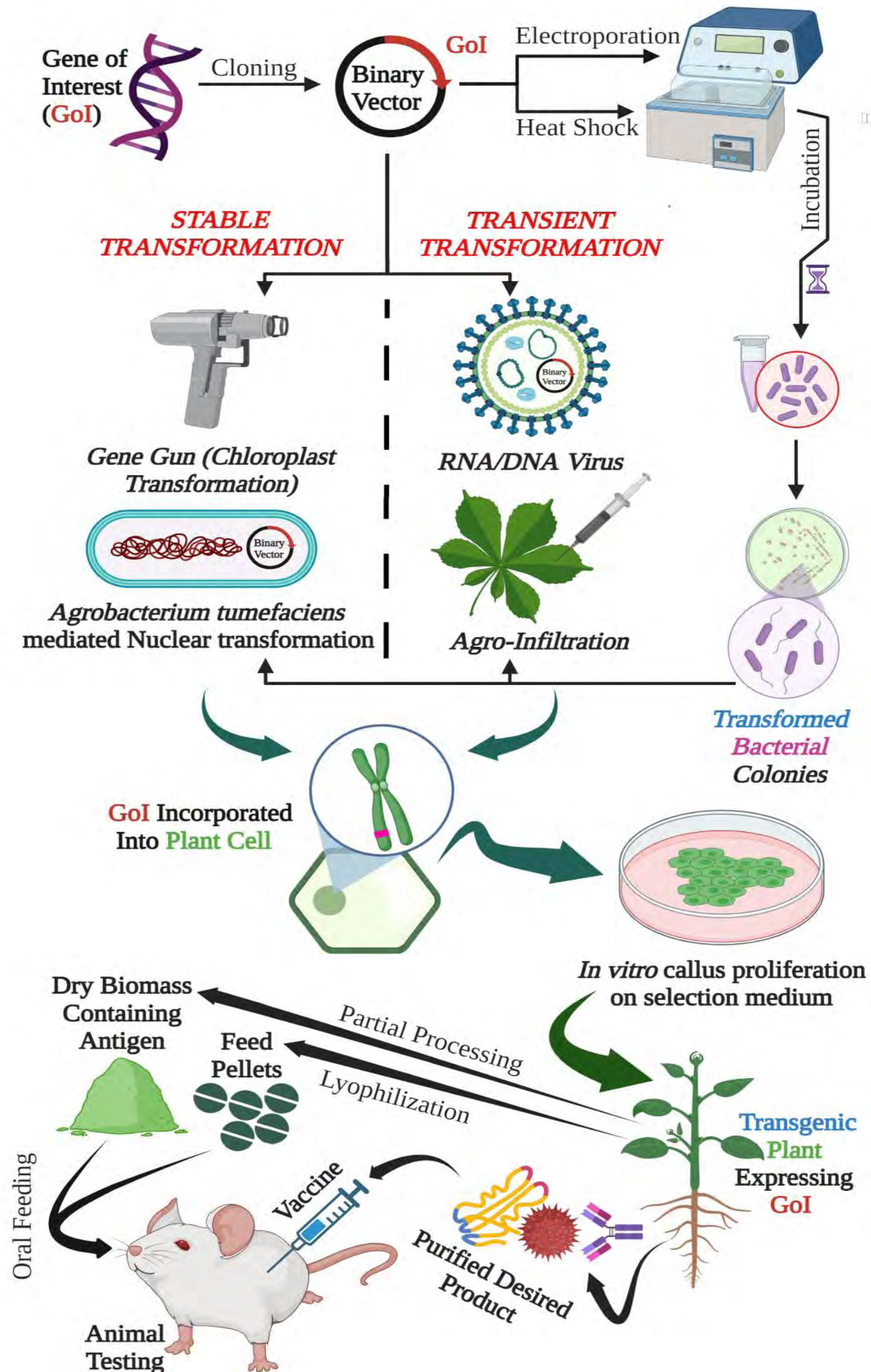



**Figure 1.2:** The processes involved in the production of plant-based vaccines.

- **Subunit vaccines:** Subunit vaccines are a type of vaccine that contains selected non-infectious components of a pathogen to elicit an immunological response. They are frequently produced recombinantly and regarded as safe. However, they may require additional components or multiple doses to be effective. Plant-based subunit vaccines have been developed for diseases such as polio, West Nile virus, rabies, anthrax, and COVID-19 (Q. Chen & Lai, 2013; Kumari *et al*., 2023) New subunit vaccines have been successfully synthesized in *N. benthamiana* such as ORF1 against cardiomyopathy syndrome (PMCV) and VLP against nervous necrosis virus (NNV) aiming to protect salmonids and Atlantic cod from these diseases (Marsian *et al*., 2019; H. Su *et al*., 2021).

### 1.5.3. Plant-based edible vaccines

Plant-based edible vaccines are recombinant protein vaccines in which selected antigens are produced by certain plant species and administered orally in the form of an edible vaccine against particular infections (Lössl & Waheed, 2011; Naik, 2022). Utilizing edible crops as green factories for producing vaccines and therapeutic proteins offers significant advantages for oral immunization. Oral vaccination of fish using edible crops or microalgae is a stress-free, convenient, and efficient strategy suitable for fish of all sizes (Clarke *et al*., 2013). A comparison of vaccination methods for fish is given in Table 1.3.

There is a limited number of studies for the expression of immunogenic antigens as veterinary vaccines in edible crops, with only one report of an oral vaccine for fish synthesized in potatoes. Further research is required for plant-based oral vaccines to be cost-effective for effective management of fish health (Clarke *et al*., 2013; Kolotilin *et al*., 2014; Shahid & Daniell, 2016). Plastid genome engineering has been successfully applied to lettuce, tomato, potato, and cabbage, among others, but there have been no reports on the development of fish vaccines by expressing immunogenic antigens in edible crops using this technology. Expanding research in this area has the potential to enhance the sustainability of fish food production in the future (Boyhan & Daniell, 2011; Cardi *et al*., 2010; Clarke & Daniell, 2011; Davoodi-Semiromi *et al*., 2010; Kanagarajan *et al*., 2012; Lakshmi *et al*., 2013; Ruhlman *et al*., 2010; Shahid & Daniell, 2016).

**Table 1.3:** Comparison of different methods for fish vaccination.

| Method | Advantages | Disadvantages |
|---|---|---|
| Injection | Can be done automatically | Anesthesia is required |
| | A small amount is enough | Stress full and costly |
| | Long-time and effective protection | Need skilled labor |
| Immersion | Easy to operate | Applicable to conc. fish stocks |
| | Suitable for all-size fishes | Requires booster amount of vaccine |
| | Better immunogenic effect | No versatility |
| | Less stressful | A high vaccine dosage is required |
| Oral | Best vaccination method | |
| | Activates intestinal mucosal immunity | Can easily be destroyed by the digestive tract |
| | Simple and easy to operate for fish of all sizes | A high vaccine dosage is required |
| | Suitable for dispersed fish | Poor immunization effect |
| | No stress effect | |
| **References** | | |
| (Chettri *et al*., 2013; Huising *et al*., 2003; Ramírez-Paredes *et al*., 2019; Tobar *et al*., 2011; Weiner *et al*., 2011; W. Xu *et al*., 2019; Y.-Y. Yao *et al*., 2019; C. Zhang *et al*., 2018) | | |

## 1.6. Reverse vaccinology (RV)

Bioinformatics provides an excellent chance for predicting the immunological and physiochemical aspects of a vaccine construct, as well as its safety and efficacy, allowing scientists to create a vaccine that is both effective and safe for use in animals (Parvizpour *et al*., 2020). With recent developments in bioinformatics, vaccine development now has access to a plethora of new and vastly better techniques (Baliga *et al*., 2018).

Computational vaccinology, also known as *in silico* vaccine designing, is an innovative approach that utilizes bioinformatics-based computational tools and software to design vaccines (Arora & Aryandra, 2020). This field has witnessed the development of various software and tools for constructing and predicting the potential properties of vaccines (Oli *et al*., 2020). Among the approaches used in computational vaccinology, reverse vaccinology (RV) and structure vaccinology (SV) are prominent methods for the robust development of vaccines (N. Yang *et al*., 2021).

Additionally, the most recent progress in safe and effective reverse vaccinology (RV) necessitates the identification of immunogenic, non-toxic, and safe substances that can elicit cell-mediated and humoral immunity (Baliga *et al*., 2018). As a result, modernized web servers for precise immune-dominant component prediction have been established (Islam, Mahfuj, *et al*., 2022; Sunita *et al*., 2020).

Computational vaccinology applications have also extended to species of marine ecosystems in recent years such as *Photobacterium damselae* subsp. *piscicida*, a pathogen affecting aquatic organisms (Andreoni *et al*., 2016). This technology has also been successfully employed in designing vaccines for two important intracellular pathogens of fish, *Edwardsiella tarda*, and *Flavobacterium columnare*, which cause Edwardsiellosis and columnaris, respectively (Mahendran *et al*., 2016).

A noteworthy application of reverse vaccinology showed its potential in developing a vaccine to prevent pasteurellosis in aquaculture, specifically targeting *Pasteurella atlantica* in lumpfish (*Cyclopterus lumpus*) (Ellul *et al*., 2021). Through an *in silico* and functional evaluation promising gene targets, particularly outer membrane proteins, can be identified that could be prioritized for subunit vaccine development, aiming to prevent disease outbreaks caused by pathogens (H. Mondal & Thomas, 2022).

### 1.7. Outer membrane protein K (OmpK) as subunit vaccine

Gram-negative bacteria are characteristically surrounded by an outer membrane that contains unique constituents known as outer membrane proteins (OMPs). OMPs are responsible for selective permeability and the integrity of the bacterial outer membrane. The immunological defense mechanism of the host can easily identify them as foreign antigens and OMPs could efficiently activate the host's acquired immune response due to their localization at the bacterial cell surface. In recent years, for the development of subunit vaccines, the OMPs have been attractive molecules due to their high immunogenicity, conserved nature, and exposed epitopes (Hamod *et al*., 2012; Heenatigala *et al*., 2020).

Different studies have shown that in *Vibrio* spp. the outer membrane protein K (OmpK) is highly conserved and can act as a promising candidate for vaccine production due to its immunogenic nature against fish vibriosis among the various OMPs identified. OMPs such as OmpV, OmpW, and LamB have gained attention as vaccine candidates (Hamod *et al*., 2012; N. Li *et al*., 2010; Lun *et al*., 2014; Mao *et al*., 2007; B. Peng *et*

*al*., 2016; W. Wang, Sang, *et al*., 2021; Xing *et al*., 2017). Furthermore, different reports show discrepancies in the specificity of OmpK antibodies for diagnostic purposes. The immunogenicity of soluble OmpK is still unknown, as most studies have focused on expressing it as inclusion bodies (N. Li *et al*., 2010; Y. Li *et al*., 2010; W. Wang, Guo, *et al*., 2021).

Several studies explored oral vaccines against *Vibrio* species in fish. Mao *et al*. (2011) developed an oral vaccine by expressing OmpK in yeast, protecting Japanese sea bass. Galindo-Villegas *et al*. (2013) enhanced a commercial vaccine for *V. anguillarum* in sea bass with rTNFα (as adjuvant), improving immune responses. Sarropoulou *et al*. (2012) evaluated a commercial vaccine in European sea bass, revealing a gene involved in gut immune responses. Gao *et al*. (2016) developed chitosan nanoparticles for oral vaccination in turbot that significantly increased specific antibodies. Li *et al*., (2013) used a DNA vaccine in chitosan nanoparticles which protected black seabream. Li *et al*. (2015) showed partial protection in gold-lined/silver sea bream because of formalin-killed *V. alginolyticus*. These studies highlight the potential of oral vaccines in fish species, providing immune responses and protection against vibriosis, with implications for the aquaculture industry.

Although the research on using plants for producing subunit vaccines for fish is limited, as it is a growing field (Marsian *et al*., 2019; H. Su *et al*., 2021). Utilizing plants for oral fish vaccines holds significant promise for the aquaculture industry. One advantage of recombinant subunit vaccines produced through plants is that it has the potential to provide simultaneously multiple antigen proteins (Buyel, 2019). Still, as of now, no vaccine for fish produced through plants has been commercialized (H. Su *et al*., 2021). Therefore, further research is crucial to advance fish vaccine production through plant biotechnology.

### 1.8. Aims and Objectives

The present study had the following aims and objectives:

- ✓ To utilize various bioinformatics tools and approaches to analyse the characteristics of the OmpK protein for designing a vaccine.
- ✓ To establish the stable expression of the OmpK antigen in tobacco plants, aiming to develop a plant-based vaccine against vibriosis for application in aquaculture.

- ✓ To achieve transient expression of the OmpK antigen in the edible plant species *Lactuca sativa*, exploring the potential for developing a plant-based edible vaccine against vibriosis.
- ✓ To assess the immunogenic potential of recombinant OmpK protein derived from plants *in vivo* using mice and fish as animal models.

The flowchart (Figure 1.3) illustrates a hierarchical approach employed in this study to accomplish the desired objectives related to the OmpK protein.

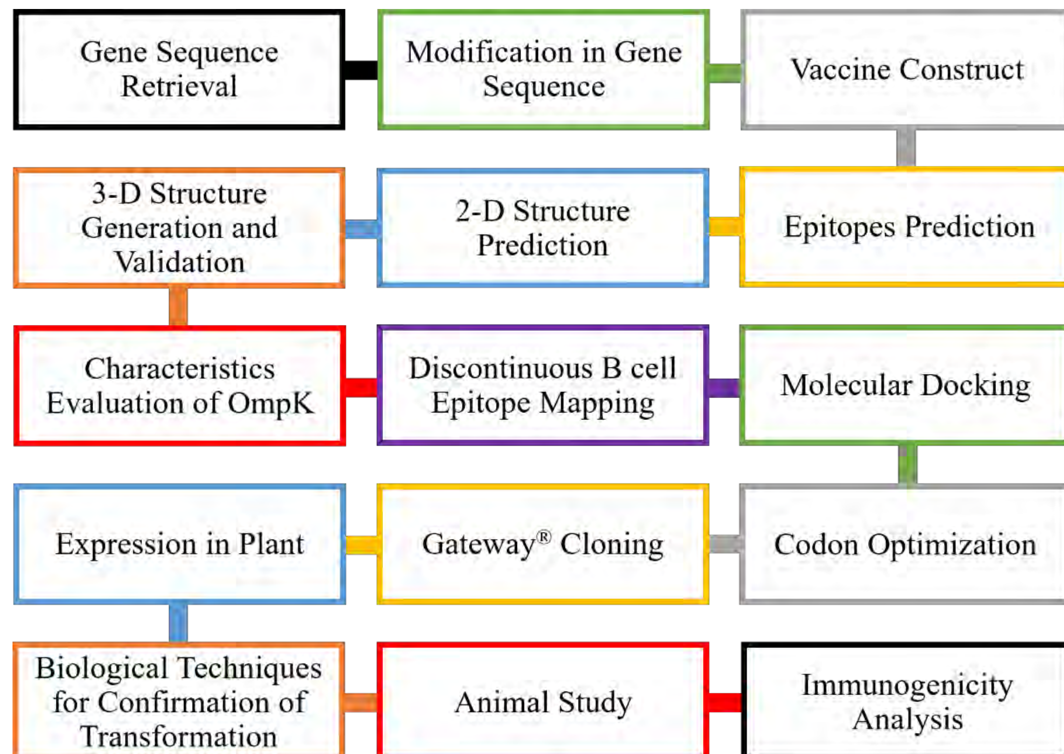



**Figure 1.3:** The hierarchical approach employed in this study.

# *MATERIALS AND METHODS*

*Chapter 2*

## 2.1. Materials

**Table 2.1:** Chemicals and reagents

| Chemicals | Catalog No. |
|---|---|
| Acetosyringone ($C_{10}H_{12}O_4$) | Sigma-Aldrich®, Saint Louis, USA |
| Agar plant TC (Micropropagation Grade) | Phytotechnology Laboratories®, US |
| Agarose | Sigma-Aldrich®, Saint Louis, USA |
| Acrylamide ($C_3H_5NO$) | Research Products International, US |
| Ammonium persulfate ($(NH_4)_2S_2O_8$) | Sigma-Aldrich®, Saint Louis, USA |
| Bacteriological agar | PlantMedia™ Dublin, Ohio, USA |
| Bacto-Tryptone | Research Products International, US |
| Bicine ($C_6H_{13}NO_4$) | PR1MA™_MIDSCI™, Fenton, UK |
| Bis-Tris ($C_8H_{19}NO_5$) | Sigma-Aldrich®, Saint Louis, USA |
| Boric acid ($H_3BO_3$) | Scharlab, Barcelona, Spain |
| Bovine serum albumin [BSA] ($C_8H_{21}NOSi_2$) | MP Biomedical, USA |
| Bromophenol Blue ($C_{19}H_{10}Br_4O_5S$) | Sigma-Aldrich®, Saint Louis, USA |
| Cetyltrimethylammonium bromide [CTAB] ($[(C_{16}H_{33})N(CH_3)_3]Br$ | Oxford lab chem, India |
| Chlorbutanol ($C_4H_7Cl_3O$) | Produits Dentaires SA, Swizerland |
| Chloroform ($CHCl_3$) | Sigma-Aldrich®, Saint Louis, USA |
| Ethanol ($C_2H_6O$) | Merck, Germany |
| Ethidium Bromide ($C_{21}H_{20}BrN_3$) | Sigma-Aldrich®, Saint Louis, USA |
| Ethylene diamine tetra-acetate acid ($C_{10}H_{16}N_2O_8$) | AnalaR™, England |
| Glucose ($C_6H_{12}O_6$) | GlaxoSmithKline, UK |
| Glycerol ($C_3H_8O_3$) | AnalaR™, England |
| Glycine ($C_2H_5NO_2$) | Merck, Germany |
| Isoamyl alcohol ($C_5H_{12}O$) | AnalaR™, England |
| Luria-Bertani (Miller) | Microgen, India |
| Luria-Bertani agar | Research Products International, US |
| Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) | AnalaR™, England |
| Methanol ($CH_3OH$) | Sigma-Aldrich®, Saint Louis, USA |
| Murashige and Skoog (MS) | Phytotechnology Laboratories®, US |
| *N,N'*-(1,2-Dihydroxyethylene)bisacrylamide ($C8H_{12}N_2O_4$) | Sigma-Aldrich®, Saint Louis, USA |
| Phenol ($C_6H_6O$) | Merck, Germany |

| | |
|---|---|
| Polyvinylpyrrolidone [PVP] $(C_6H_9NO)_n$ | Research Products International, US |
| Potassium chloride (KCl) | AnalaR™, England |
| Propa-2-ol ($C_3H_8O$) | AnalaR™, England |
| Skim milk powder | Scharlab, Barcelona, Spain |
| Sodium dodecyl Sulfate [SDS] ($NaC_{12}H_{25}SO_4$) | Phtotechnology Laboratories, USA |
| Sodium acetate ($CH_3COONa$) | AnalaR™, England |
| Sodium azide ($NaN_3$) | Research Products International, US |
| Sodium bicarbonate ($NaHCO_3$) | Research Products International, US |
| Sodium carbonate ($Na_2CO_3$) | Research Products International, US |
| Sodium chloride (NaCl) | Applichem, USA |
| Sodium hydroxide (NaOH) | Merck, Germany |
| Sucrose ($C_{12}H_{22}O_{11}$) | AnalaR™, England |
| Sulphuric acid ($H_2SO_4$) | Sigma-Aldrich®, Saint Louis, USA |
| Tetramethylethylenediamine [TEMED] ($C_6H_{16}N_2$) | Research Products International, US |
| Tris-(hydoxymethyl) aminomethane [Tris base] ($C_4H_{11}NO_3$) | Invitrogen, USA |
| Tris-(hydroxymethyl) aminomethane hydrochloride [Tris HCl] ($C_4H_{13}Cl_2NO_3$) | Phtotechnology Laboratories, USA |
| Tween-20 ($C_{58}H_{114}O_{26}$) | Sigma-Aldrich®, Saint Louis, USA |
| Yeast extract | Research Products International, US |
| β-mercaptoethanol ($C_2H_6OS$) | Biochem, USA |

**Table 2.2:** Ready-to-use reagents and kits

| Reagents | Company |
|---|---|
| 6x-His-tag monoclonal antibody | Invitrogen, USA |
| Chemiluminescent HRP substrate | Millipore, USA |
| Complete protease inhibitor | Sigma-Aldrich®, Saint Louis, USA |
| dNTP Set (100mM) | Thermofisher, USA |
| Gateway™ BP Clonase™ II Enzyme mix | Invitrogen, USA |
| Gateway™ LR Clonase™ II Enzyme mix | Invitrogen, USA |
| GeneJet gel extraction kit | Thermofisher, USA |
| Gene Ruler™ 1 kb DNA ladder | Thermofisher, USA |
| Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG | Abcam, USA |
| Nitrocellulose Membranes, 0.45 μm | Thermofisher, USA |

| Protein ladder | Thermofisher, USA |
|---|---|
| Proteinase K | Thermofisher, USA |
| RNAase A | Bioworld |
| SYBR green | Thermofisher, USA |
| *Taq* DNA polymerase (5U/µL) | Thermofisher, USA |
| TMB (3,3,5,5-tetramethylbenzidine) ELISA substrate solutions | Thermofisher, USA |

**Table 2.3:** Hormones and antibiotics

| **Hormones** | **Company** |
|---|---|
| 6-Benzylaminopurine [BAP] ($C_{12}H_{11}N_5$) | Research Products International, US |
| Indole-3- acetic acid [IAA] ($C_{10}H_9NO_2$) | Sigma-Aldrich®, Saint Louis, USA |
| Myo-inositol ($C_6H_{12}O_6$) | Research Products International, US |
| Thiamine HCl ($C_{12}H_{18}C_{12}N_4OS$) | Sigma-Aldrich®, Saint Louis, USA |
| **Antibiotics** | **Company** |
| Ampicillin ($C_{16}H_{18}N_3NaO_4S$) | Research Products International, US |
| Cefotaxime ($C_{16}H_{16}N_5NaO_7S_2$) | Sanofi-aventis, Pakistan |
| Hygromycin B ($C_{20}H_{37}N_3O_{13}$) | Thermofisher, USA |
| Kanamycin ($C_{18}H_{36}N_4O_{11}.H_2SO_4$) | Carl Roth®, Germany |
| Rifamycin ($C_{37}H_{47}NO_{12}$) | Research Products International, US |
| Tetracycline HCl ($C_{22}H_{25}ClN_2O_8$) | Research Products International, US |

**Table 2.4:** Primers and probes

| **Primers** | **Sequences (5′to3′)** |
|---|---|
| OmpK Nuclear Forward (OmpK-NF) | GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATG GTAAATCACTTTTAGCTCTAGGCC |
| OmpK Nuclear Reverse (OmpK-NR) | GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGT GATGGTGATGGTGATG |
| OmpK Internal Forward (OmpK-IF) | CTAAGCAACCCAAGCAGTGACAAAG |
| OmpK Internal Reverse (OmpK-IR) | CCTTCATCTTTCAGACCATAAACATCTTTGTAGC |
| GFP Forward (GF) | ATAATGCCACCTACGGCAAGCT |
| GFP Reverse (GR) | ATATGCTCAGGTAGTGGTTGTCGG |
| *β*-actin Forward (*β*-F) | AGGTGCCCTGAGGTCTTGTTCC |
| *β*-actin Reverse (*β*-R) | ATCAGCAATACCAGGGAACATAGT |

**Table 2.5:** Primers set, their Tm, and purpose

| Set | Primers | Annealing Temp. (°C) | PCR band (bp) | For confirmation of: |
|---|---|---|---|---|
| **A.** | OmpK IF and IR | 56 | 500 | *OmpK* gene and qRT-PCR |
| **B.** | GF and GR | 62.1 | 507 | *OmpK* gene in bacteria and plants. |
| **C.** | OmpK NF and NR | 66 | 870 | |
| **D.** | OmpK IF and GR | 57 | 1230 | |
| **E** | OmpK NF and GR | 56 | 1495 | |

**Table 2.6:** Laboratory instruments

| Appliances | Company |
|---|---|
| Autoclave | Yamato, USA |
| Balance | Ohaus Corp, USA |
| Centrifuge | Eppendorf, Germany |
| MicroPulser Electroporator 1652100 | Biorad, USA |
| Freezer | Dawlance, Pakistan |
| Freezer (-70°C) | VWR, USA |
| Gel casting device | Cleaver Scientific, USA |
| Hot plate stirrer | IKA Labortechnik, Germany |
| Micropipettes | Eppendorf, Germany |
| Magnetic stirrer | VWR, USA |
| Microtiter plate reader | Bio-Rad, Japan |
| Minitron incubator | VWR, USA |
| Orbital shaker | VWR, USA |
| Semi-dry blotter | Cleaver Scientific, USA |
| Spectrophotometer (Multiskan® GO) | ThermoScientific, USA |
| Power supply | Biometra, USA |
| XCell4 Surelock™ Midi Cell | Invitrogen, USA |
| Vortex | Scientific Industries, USA |
| Water bath (Precision, 180 Series) | ThermoScientific, USA |
| Conventional PCR | Applied Biosystems, USA |
| Real-Time PCR (MyGo Pro®) | Novacyt Group, UK |
| Colibri microvolume spectrometer | Titertek Berthold, Germany |
| Gel documentation system | FluorChem™ FC3_Biotechne®, US |
| Vertical Gel electrophoresis apparatus | Cleaver Scientific, USA |

**Table 2.7:** Consumables

| Consumables | Company |
|---|---|
| 1-3 mL Plastic disposable syringes | Amson Vaccines & Pharma, Pakistan |
| 1.5-2 mL Eppendorf tubes | Axygen Scientific, USA |
| 15/50 mL Falcon tubes | Corning® USA |
| Glassware | Pyrex, USA |
| Gloves | Qualtex Malaysia |
| Microtiter plates | Costar, USA |
| Nitrile gloves | Gen-X, Malaysia |
| Parafilm PM-96 | Bemis, USA |
| PCR tubes | ThermoScientific, USA |
| Pipette Tips | Extra Gene, USA |
| Plastic feeding tubes, 18ga (30mm) | Instech Laboratories, Inc, USA |
| Surgical blades | XINDA, China |
| Syringe filters (0.2 µm) | Sartorius AG, Germany |
| Whatman filter paper | GE Healthcare, UK |

## 2.2. Methods

### 2.2.1. Culture media

Murashige and Skoog (MS) media were used for the germination of seeds of tobacco, lettuce, and spinach regeneration of plants also took place on an MS medium containing specifically optimized plant growth regulators. For bacterial inoculation and bacterial streaking, Luria-Bertani (LB), and Luria-Bertani agar (LA) media were used, respectively.

#### 2.2.1.1. Luria-Bertani (LB) media

For bacterial growth, Luria-Bertani (LB) media was used. LB was weighed (Annexure 2.1) and dissolved in the required amount of water in a flask. The flask was covered with aluminum foil and paper tape for sealing. The medium was sterilized through autoclaving at standard conditions and stored at a temperature of 25°C.

#### 2.2.1.2. Luria-Bertani agar (LA) media

LB media and agar were weighed (Annexure 2.1) and dissolved in the required amount of sterile water in a flask. The flask was covered, and the media was sterilized through autoclaving at standard conditions (121°C temperature, 15 psi pressure for 20 minutes). It was placed in a laminar flow hood and after some time the required concentration of antibiotic was added for selection. Media was poured into Petri plates (20 mL). Plates were parafilm sealed and stored in the growth room at 25°C.

#### 2.2.1.3. MS media

For the preparation of half MS media, sucrose, plant agar, and MS were weighed (Annexure 2.1) and dissolved in the required amount of water in a reagent bottle. The medium was sterilized through autoclaving at standard conditions. While it was slightly hot, calculated concentrations of antibiotics and hormones were added to the media and half MS in Petri plates (20 mL) or glass jars (50 mL). Plates and jars were sealed by parafilm and stored in the growth room.

### 2.2.2. Preparation of stock solutions of plant hormones

Different hormones were used in this research and the following are their compositions.

**2.2.2.1. 6-Benzylaminopurine (BAP)**

We prepared a 1 mg/mL stock solution of BAP and for its solubility, we used 1 M of NaOH because it is insoluble in water. We weighed 100 mg powder of BAP and added 1-2 mL of NaOH solution to completely dissolve it. After complete dissolving, its volume was raised to 50 mL with autoclaved water and sterilized using a membrane filter having a 0.2 µm filter pore size. After filtration, aliquots were made and kept at -20°C.

**2.2.2.2. Indole-3-acetic acid (IAA)**

We prepared 1 mg/mL stock solution of IAA by weighing 50 mg of IAA and dissolved it in 1-2 mL of 1 M of NaOH solution because it is insoluble in water. After complete dissolving, its volume was raised to 50 mL with autoclaved sterilized water. The stock was filter sterilized with filter assembly using a 0.2 µm filter. Aliquots were prepared after filtration and stored at -20°C for further use.

**2.2.2.3. Myo-inositol**

For the preparation of 100 mg/mL stock solution of Myo-inositol, 10 gm of Myo-inositol was weighed and dissolved in 50 mL of autoclaved sterilized water until it became transparent, then raised the volume to 100 mL. We filter-sterilized this stock solution by using a filter assembly and a sterilized syringe. The pore size of the filter paper was 0.2 µm. After filtration, aliquots were made and stored at a temperature of -20°C.

**2.2.2.4. Naphthalene acetic acid (NAA)**

We prepared 1 mg/mL stock of NAA by weighing 50 mg NAA and dissolved it in 1-2 mL of 1M of NaOH solution because it was insoluble in water. After complete dissolving, its volume was raised to 50 mL with autoclaved sterilized water. The stock was filter sterilized with filter assembly. After filtration, aliquots were made and stored at a temperature of -20°C.

**2.2.2.5. Thiamine HCl:**

For the preparation of 1 mg/mL stock solution of Thiamine HCl, 50 mg of Thiamine HCl was weighed and dissolved in 20 mL of autoclaved sterilized water until it became transparent, then raised the volume to 50 mL. We filter sterilized this stock solution by

using a filter assembly with a 0.2 µm filter and sterilized syringe. After filtration, aliquots were made and stored at a temperature of -20°C.

### 2.2.3. Preparation of stock solutions of antibiotics

Different antibiotics were used in this research and the following are their compositions.

#### 2.2.3.1. Ampicillin

To prepare the stock solution (50 mg/mL) of ampicillin, 5 gm of ampicillin was weighed and solubilized in sterile water (20 mL) until it became transparent and then raised the volume to 100 mL. The stock solution was filter sterilized with filter assembly, aliquots were made and stored at a temperature of -20°C.

#### 2.2.3.2. Hygromycin

For the preparation of 50 mg/mL stock of hygromycin, 2.5 gm of hygromycin was solubilized in 10 mL sterile water until it became transparent, then the volume was raised to 50 mL. The stock was filter sterilized with filter assembly. Aliquots were prepared after filtration and stored at -20°C for further use.

#### 2.2.3.3. Kanamycin

To prepare kanamycin stock solution having a concentration of 50 mg/mL, 5 gm kanamycin was solubilized in 20 mL sterile water until it became transparent, then raised the volume to 100 mL. The stock was filter sterilized with filter assembly using membrane filter 0.2 µm filter pore size. After filtration, aliquots were made and stored at a temperature of -20°C.

#### 2.2.3.4. Rifamycin

To prepare a 20 mg/mL stock of rifamycin, 20 mg rifamycin was solubilized in 10 mL 100% ethanol until it became transparent. The stock was filter sterilized with filter assembly using membrane filter 0.2 µm filter pore size. After filtration, aliquots were made and stored at a temperature of -20°C.

#### 2.2.3.5. Tetracycline

To prepare tetracycline stock solution having a concentration of 50 mg/mL, 5 gm of tetracycline was solubilized in 50 mL sterile water until it became transparent, then the volume was raised to 100 mL. The stock was filter sterilized with filter assembly. After filtration, aliquots were made and stored at a temperature of -20°C.

### 2.2.4. Bacterial growth conditions

All strains of bacteria (DB 3.1, DH5α, and *Agrobacterium* GV3101) were cultured either on LA plates with 1gm/100mL bacterial agar or in 50 mL liquid LB broth (Maniatis *et al*., 2003) supplemented with suitable antibiotics for selection at 28ºC for *Agrobacterium* strain and at 37ºC for all other bacterial strains. For bacterial growth in LB liquid media, it was incubated in a shaking incubator until it attained a 0.6-0.8 optical density (OD) value. The OD value was measured by using a spectrophotometer at 600nm. The working concentrations of different antibiotics for transformed bacteria selection are given in Table 2.8.

**Table 2.8:** Working concentrations of different antibiotics

| Antibiotics | Working concentration (mg/L) |
|---|---|
| Ampicillin | 100 |
| Kanamycin | 50 |
| Rifamycin | 100 |
| Tetracycline | 10 |

### 2.2.5. Bacterial transformation

The following processes were carried out for the transformation of bacteria.

#### 2.2.5.1. Electro-competent cells' preparation

In this study, we prepared the competent cells of DB 3.1, DH5α, and *Agrobacterium* GV3101. To prepare competent cells, the bacterial culture was shifted to the falcon tube (50 mL). The centrifugation was carried out at a speed of 4,000 rpm for 20 minutes at 4°C. Resuspended the pellet in distilled water (40 mL) and mixed it thoroughly after decanting the supernatant. The mixture was again centrifugated at a speed of 2,400 rpm for 20 minutes at 4°C. The pellet was resuspended and thoroughly mixed in distilled water (20 mL) by decanting the supernatant once again. After that, it was centrifuged for the third time under the above-mentioned conditions. This time the obtained pellet was dissolved in an ice-cold-distilled 10% glycerol (10 mL) after decanting the supernatant and centrifuged for a fourth time at a speed of 2,400 rpm for 20 minutes at 4°C. Resulted pellet, after decanting the supernatant, was subjected to the addition of an ice-cold-distilled 10% glycerol (500 µl). 50 µL aliquots were made and stored at the temperature of -70°C.

### 2.2.5.2. Electroporation

All plasmids were integrated into electro-competent cells via electroporation by following the manufacturer's protocol using Electroporator. 50 µL of ice-thawed electro-competent cells and 1 µL of plasmid were thoroughly mixed and transferred into a cuvette. The electroporation conditions were set as described in the manual and pressed the pulse button. 250 µL SOC media (Annexure 2.2) was added in a cuvette and mixed gently. Afterward, it was put into an Eppendorf tube and incubated for 2-3 hours with gentle shaking at a temperature of 37°C. For the selection of transformed bacteria, 50 µL of bacterial cultures were streaked on LA plates enriched with corresponding suitable antibiotics, and subsequently incubated overnight at a temperature of 37°C.

### 2.2.6. DNA isolation

DNA from bacterial and plant cells or tissues is isolated by following the mentioned protocols.

### 2.2.6.1. Plasmid DNA isolation

Plasmid DNA was extracted by following the procedure outlined by Sambrook and Rusell (2001) with certain modifications. The bacterial culture having the $OD_{600}$ value between 0.6-0.8 was shifted to the falcon tube (50 mL) for isolation of plasmid DNA. The falcon tube was centrifugated at a speed of 4,000 rpm for 10 minutes at 4°C. The resulting bacterial pellet was recovered by discarding the upper clear solution. The pellet was dissolved in 200 µL solution I (Annexure 2.3) by vortexing. After this, the dissolved pellet was transferred to a microcentrifuge tube. Then 400 µL solution II (Annexure 2.3) was added to the dissolved pellet. The microcentrifuge tube was gently inverted for proper dissolution of the pellet and then for 3-5 minutes placed on ice. Then 300 µL of solution III (Annexure 2.3) was added and gently inverted to properly mix the solutions followed by storage on ice for 5 minutes. To collect supernatant, the microcentrifuge tube was then centrifugated at 4°C for 5 minutes by setting the speed of 14,000 rpm. The 600 µL of supernatant was pipetted into a fresh tube. To that supernatant, 600 µL of phenol-chloroform (Annexure 2.3) was added. The mixture was gently inverted 6-7 times for proper mixing and then centrifugated at a temperature of 4°C for 5 minutes at a maximum speed of 14,000 rpm. The upper plasmid-containing layer was collected in a fresh microcentrifuge tube followed by 600 µL of isopropanol

addition. The tube was incubated on ice for one hour. After incubation, it was centrifuged at 4°C for 5 minutes at 14,000 rpm. The pellet was dried after decanting the supernatant and rinsed with 70% ethanol (1 mL) followed by centrifuging at 14,000 rpm for a total of 2 minutes. Ethanol was decanted and the pellet was dried. Pellet was then dissolved in 20-30 µL TE buffer + RNAase A (Annexure 2.3) according to the size of the pellet and stored at -20°C.

#### 2.2.6.1. Plant genomic DNA isolation

Genomic DNA of the plant was isolated by following the cetyltrimethylammonium bromide (CTAB) protocol as reported by Murray and Thompson (1980). DNA was extracted from the leaves of both transformed and wild-type plants. During extraction, liquid nitrogen was used to freeze the leaves before they were pulverized into fine powder with pestle mortar. Then 700 µL CTAB buffer (Annexure 2.4) (pre-heated for 30 minutes in a water bath at 65°C) was added. The samples were subjected to incubation in the water bath for an hour at 65°C. After incubation, the samples were cooled down to room temperature. Afterward, the 700 µL of chilled phenol-chloroform-isoamyl alcohol (Annexure 2.4) was pipetted to the sample and gently inverted for 30 minutes for proper mixing of the two solutions. Then the mixture was subjected to centrifugation at 10,000 rpm for 15 minutes. To a new sterilized Eppendorf tube, the supernatant was transferred. Finally, in the sample, chilled isopropanol (1 mL) was added for the precipitation of DNA. The Eppendorf tube was subjected to centrifugation at 10,000 rpm for 10 minutes. After discarding the supernatant, the DNA pellet was rinsed twice with 70% ethanol (Annexure 2.4) and subjected to centrifugation at 10,000 rpm at a temperature of 20°C for 5 minutes. The collected pellet was then air dried by placing it inverted on a filter paper towel for 20 minutes. The pellet was then dissolved by adding the proper amount of TE buffer + RNAase A (Annexure 2.3) according to pellet size and was stored at the temperature of -20°C.

### 2.2.7. DNA concentration quantification

To quantify the concentration of DNA in ng/µL, a Colibri microvolume spectrometer (Titertek Berthold, Germany) was used.

### 2.2.8. Gel electrophoresis

DNA size, PCR amplicon, and restriction fragment confirmation were done through agarose gel electrophoresis. For the preparation of 1% agarose gel, 1 gm of agarose was

weighed and mixed with 1X TBE buffer (100 mL) (Annexure 2.5). The solution was then heated for 2 minutes in an oven to properly dissolve the agarose. It was brought to room temperature and 7 µl ethidium bromide (Annexure 2.5) was added. A comb was inserted into the casting tray for well-formation followed by the pouring of gel. 3 µL loading dye per 7 µL sample was added and DNA was separated at 120 V using a horizontal gel electrophoresis system.

### 2.2.9. Gel extraction

For purification of PCR product and restriction fragments before cloning, gel extraction was carried out according to the GeneJet gel extraction kit. The part of agarose gel containing the band was cut and weighed (approximately 100 mg) and this piece of gel was transferred to a sterilized Eppendorf tube. 300 µL HUB buffer was pipetted to it and then subjected to incubation for 10 minutes at 60°C. Then dissolved mixture from the previous step was put into a Mini spin column followed by centrifugation for just 30 seconds at a speed of 7,000 rpm. The flow-through was decanted followed by the pipetting of 80% ethanol (750 µL) and centrifugation for 3 minutes duration at 13,000 rpm. Flow-through was again decanted and the DNA was eluted with elution buffer (30-50 µL) and stored at a temperature of -20°C.

### 2.2.10. Transgene integration confirmation via PCR

Confirmation of transgene integration within the transformed tobacco plants and transiently transformed spinach and lettuce plants was done via PCR. The Cetyltrimethylammonium bromide (CTAB) method proposed by Murray and Thompson (1980) was used to extract DNA from wild-type and putative-transformed plant. The details of the protocol are given previously. Tables 2.9 and 2.10 represent the PCR conditions employed in this study.

**Table 2.9:** PCR conditions

| Steps | Temperature (°C) | Time (mins: secs) | |
|---|---|---|---|
| **Initial denaturation** | 95 | 03:00 | |
| **Template denaturation** | 95 | 00:40 | 35 cycles |
| **Annealing of primers** | Variable | 01:00 | |
| **Extension of new DNA strands** | 72 | Variable | |
| **Final extension** | 72 | 10:00 | |
| **End** | 4 | Indefinite | |

**Table 2.10:** PCR 1X Master mix composition

| Reagents | Concentration (µL) |
|---|---|
| 10X Buffer | 2.5 |
| 25mM $MgCl_2$ | 1.5 |
| Double distilled $H_2O$ | 18 |
| DNA template | 1.0 |
| DNTPs mix | 0.5 |
| Forward primer | 0.5 |
| Reverse primer | 0.5 |
| Taq DNA polymerase (5U/µL) | 0.5 |

### 2.2.11. Western blot analysis

Protein detection and quantification in the plant samples were performed through Western blotting. The following steps were involved.

#### 2.2.11.1. Protein extraction

The protocol of protein extraction is given in the Materials and Methods section of each Chapter separately.

#### 2.2.11.2. Sample preparation

Each sample was prepared by adding 1X reducing agent and 4X loading dye in the plant protein sample and subjected to heating for 10 minutes at 95°C. The standard curve was drawn through Bradford assay using known dilutions of bovine serum albumin (Annexure 2.6) for estimation of total protein content in a sample.

#### 2.2.11.3. Gel preparation

The gel for protein separation was prepared by the Cleaver Scientific Ltd. protocol with little modification. The 15% separating gel was prepared for the separation of proteins. 10 mL of 30% acrylamide stock solution (Annexure 2.7) and 5 mL of distilled water were mixed carefully. Afterward, the addition of 5 mL of 4X separating Tris-solution (Annexure 2.7) was made. To this mixture, 400 µL of 10% APS (Annexure 2.7) was added. Similarly, 5% stacking gel was prepared by mixing 2 mL of 30% acrylamide stock solution into 6.76 mL of distilled water. Then 3 mL of 4X stacking gel Tris-

solution (Annexure 2.7) was added to that mixture. Finally, 120 µL of 10% SDS (Annexure 2.7) and 120 µL of 10% APS were added to it.

**2.2.11.4. Gel pouring**

The comb was inserted into the glass plates and marked a point on them 3 cm below where the comb finished. This mark was the indication of the level for separating gel. A 37 µL of TEMED was added to the solution made for the separating gel and avoided air bubble formation during mixing. The solution was warmed a little with boiling water. The solution was added quickly into the glass plates up to the mark for separating gel and air bubble formation was avoided. So that the TEMED does not cause the solution to become too viscous and make the pouring difficult. 1 mL of isopropanol was overlayed very carefully to remove any chances for air bubble formation. The gel was allowed to get polymerize (up to 15 minutes). After the separating gel get polymerized than overlay liquid was poured off. The gel was rinsed with water. After that, the 25 µL of TEMED was added to the stacking gel solution carefully by avoiding air bubble formation. The solution was warmed also with boiling water and then carefully poured with a pipette into glass plates followed by the insertion of a comb. It was made sure that under the comb's teeth, no air bubbles were trapped as these will inhibit the progression of samples. For proper polymerization, the stacking gel was left for up to 30 minutes.

**2.2.11.5. Sample loading**

During the transfer of the inner gel module containing cast gels into the tank, the correct orientation of the inner module was maintained so that the inner gel module and tank positive and negative terminals are correctly aligned with each other. The tank was filled with 1X running buffer (Annexure 2.8) up to the mark on the tank. In each well up to 25 µL of protein sample was loaded. 3.5 µL of protein ladder was loaded for indication of the size of bands in the first well. The orientation and order in which the samples were loaded were also noted to avoid any confusion later.

**2.2.11.6. Electrophoresis**

The lid was fitted and connected to the power supply. The gel was first run for 20 minutes at 80 V so that protein samples run smoothly across the stacking gel. After the protein sample has crossed the stacking gel the voltage setting has been changed to 110 V. The power supply was turned off when the loading dye reached the bottom of the

gel. From the tank, the gel casting module was removed. Then carefully removed the gel by soaking it in TBS-T from the glass plates after unscrewing them.

**2.2.11.7. Protein transfer**

Protein was transferred onto a nitrocellulose membrane in a Semi-dry blotter module. The membrane was activated by putting it into ethanol/methanol for 30 seconds and followed by washing it with distilled water. For making the sandwich two filter papers, nitrocellulose membrane, gel, and four sponge pads were placed in 1X transfer buffer (Annexure 2.8). The arrangement of the sandwich is given in Figure 2.1. To a Semi-dry blotter module, the sandwich assembly was shifted. The modular was tightly screwed and connected to a power supply. The transfer was done at 20 volts for 75 minutes.

**2.2.11.8. Blocking**

Membrane blocking was done to avoid the antibodies' non-specific binding with the membrane. It was achieved by treating the membrane with TBS-TM (Annexure 2.8) for 20 minutes with continuous shaking at room temperature. Excess binding of skim milk with the membrane was avoided by washing it with TBS-T (Annexure 2.8) thrice.

**2.2.11.9. Primary and secondary antibodies treatment**

The membrane was allowed to react with anti-6xHis antibody (1:10000 dilution in TBS-T containing 5% BSA and 0.05% sodium azide) overnight at $4^{o}C$ with gentle agitation followed by washing three times with TBS-T. Then subjected to incubation with horse radish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (IgG) antibodies (Abcam, USA) (1:10000 dilution in TBS-T containing 5% BSA) as secondary antibody at room temperature for 1.5 hours followed by washing again with TBS-T thrice.

**2.2.11.10. Detection**

After washing, the reaction visualization was carried out by dipping the membrane in a Chemiluminescent HRP substrate and detected by a chemiluminescent system.

**2.2.12. Protein quantification**

Bradford assay was used to quantify the concentration of total extracted soluble leaf protein in a sample. A standard curve was drawn by using the Bovine serum albumin (BSA) as a standard. A stock solution of 2 mg/mL was prepared. Eight dilutions (Annexure 2.6) of 10 µl were made from stock. Then 2 µl of blank, BSA dilutions and

sample to be quantified were loaded in 96 well plates in sequence. Each sample was loaded in triplicates. In each well-containing sample, 200 µL of Bradford reagent was added followed by incubation in the dark for 30 minutes. After incubation, absorbance was noted at 595 nm using a spectrophotometer. Through absorbance values of BSA dilutions standard curve was drawn as shown in Figure 2.2 and concentrations of unknown protein were found by the TREND formula on an Excel sheet.

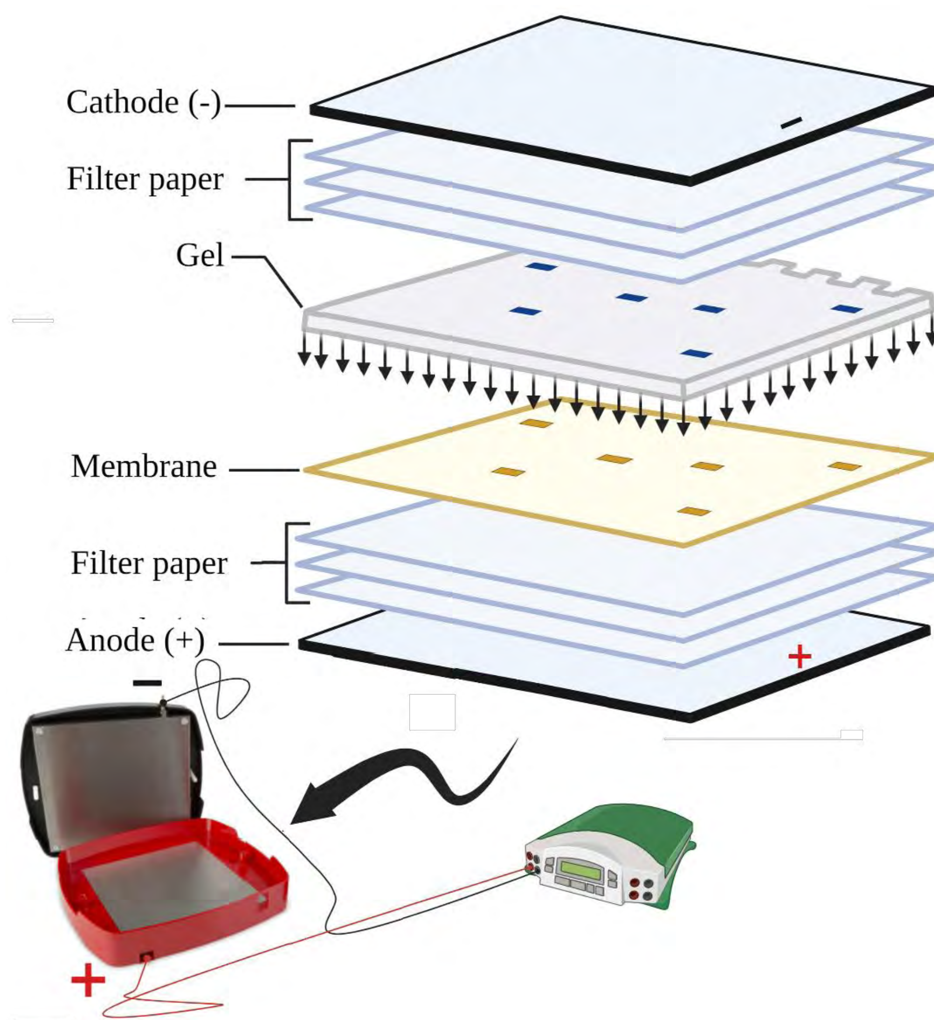



**Figure 2.1:** The arrangement of sandwich for transfer of protein in Western blotting

### 2.2.13. Enzyme-Linked Immunosorbent Assay (ELISA)

Protein extracts were prepared in ELISA extraction buffer (Annexure 2.9) from the fresh leaf tissue of transgenic and non-transgenic leaves. The homogenized sample was prepared by weighing 100 mg of leaves and grounding them in pre-chilled mortar and pestle in liquid nitrogen. To collect the supernatant (soluble protein fraction), the grind was centrifugated at 20,000 g at 4ºC for 10 minutes. The 96-well microtiter plate was

coated with proteins extracted from transformed and untransformed leaves and incubated at 4ºC overnight. The microtiter plate was subjected to washing with TBS-T thrice. Then treated with TBS-TM at 37ºC for an hour for blocking. To each well 50 µL of primary antibody (anti-6xHis antibody diluted 1:1000 in TBS) was pipetted and then subjected to incubation for an hour followed by washing with TBS-T three times. After that, each sample containing well was treated with a secondary antibody (Peroxidase-conjugated goat anti-mouse IgG:1:3000 dilution in TBS). Then the plate was subjected to incubation for an hour at a temperature of 37ºC. The plate was subjected to washing with TBS-T thrice followed by 100 µL of 3,3,5,5-tetramethylbenzidine (TMB) substrate addition in each sample containing well. To stop the reaction 100 µL of 0.16 M $H_2SO_4$ was pipetted after 10-20 minutes. Absorbance was noted at 450nm with a microtiter plate reader.

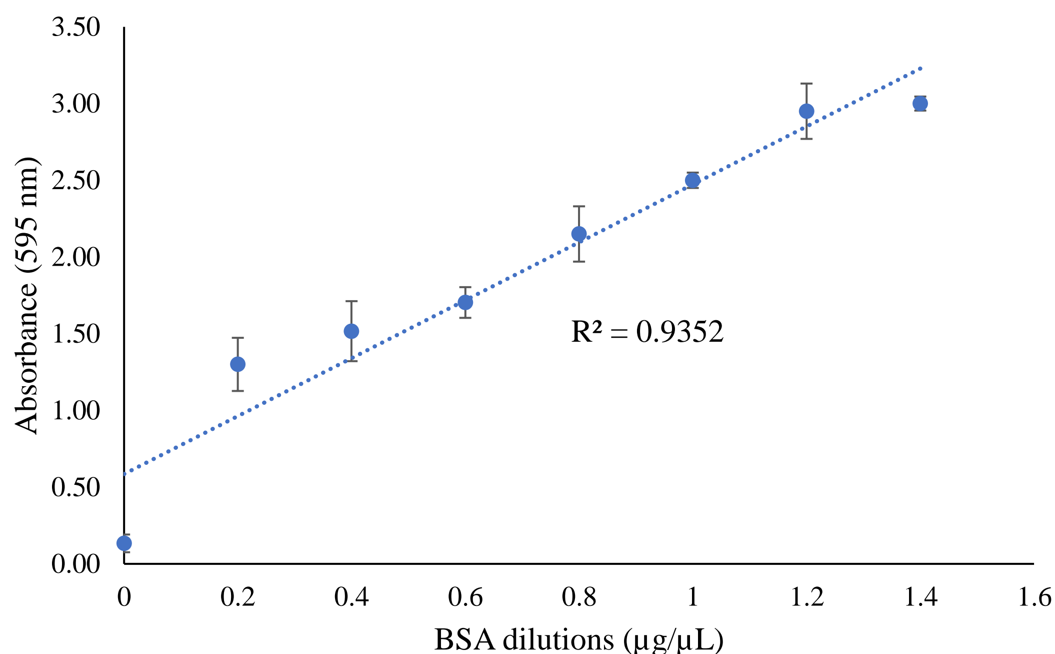



**Figure 2.2:** BSA Standard curve for protein estimation

**Annexure 2.1:**

**Reagents for culture media**

| Media | Components | Concentration (gm/L) |
|---|---|---|
| **Half MS** | MS basal salt | 2.2 |
| | Plant agar | 7 |
| | Sucrose | 30 |
| **LA** | Bacteriological agar | 10 |
| | LB broth | 25 |
| **LB** | LB broth | 25 |

**Annexure 2.2:**

**Composition of SOC media**

| Components | Concentration |
|---|---|
| Bacto-Tryptone | 20 gm/L |
| 1 M Glucose | 20 mL/L |
| 1 M KCl | 2.5 mL/L |
| 1 M $MgCl_2$ | 10 mL/L |
| NaCl | 0.5 gm/L |
| Yeast extract | 5 gm/L |

**Annexure 2.3:**

**Composition of plasmid extraction solutions**

| Solutions | Components | Concentration |
|---|---|---|
| **Solution I** | EDTA | 10 mM; pH 8 |
| | Glucose | 50 mM |
| | Tris | 25 mM; pH 8 |
| **Solution II** | NaOH | 0.2 N |
| | SDS | 1% |
| **Solution III** | Sodium acetate | 3 M pH; 4.8 |
| **Phenol-chloroform** | Chloroform | 25 mL |
| | Phenol | 25 mL |
| **TE buffer** | EDTA | 0.5 M |
| | Tris HCl pH 8.0 | 1 M |

**Annexure 2.4:**

**Solutions for plant DNA extraction**

| Solution | Compositions | Concentration |
|---|---|---|
| **CTAB buffer** | CTAB | 2% |
| | EDTA | 20mM |
| | NaCl | 1400 mM |
| | Tris HCl (pH 8) | 100mM |
| | PVP | 1% |
| | β-mercaptoethanol | 0.2% |
| **Phenol-chloroform-isoamyl** | Chloroform | 24 mL |
| | Isoamyl alcohol | 1 mL |
| | Phenol | 25 mL |
| **70 % Ethanol** | Ethanol | 70 mL |
| | Distilled Water | 30 mL |

**Annexure 2.5:**

**Gel electrophoresis**

**TBE buffer recipe 10X (pH 8.0)**

| Components | Concentration (gm/L) |
|---|---|
| Boric acid | 55 |
| EDTA | 9.3 |
| Tris base | 110 |

**Ethidium bromide preparation (10 mg/mL)**

| Components | Concentration |
|---|---|
| Ethidium bromide | 1 gm |
| Distilled water | 100 mL |

**Annexure 2.6:**

**Dilutions of BSA for Bradford reagent**

| Concentration (µg/µl) | BSA (µL) | Distilled $H_2O$ (µL) |
|---|---|---|
| 0.0 | 0 | 20 |
| 0.2 | 2 | 18 |
| 0.4 | 4 | 16 |
| 0.6 | 6 | 14 |
| 0.8 | 8 | 12 |
| 1.0 | 10 | 10 |
| 1.2 | 12 | 8 |
| 1.4 | 14 | 6 |

**Annexure 2.7:**

**Composition of Western gel preparation solutions**

**Acrylamide gel solution**

| Components 30% stock | Concentration per 100mL |
|---|---|
| Acrylamide | 30 gm |
| Methylene bisacrylamide | 0.8gm |
| Distilled water | Up to 100mL |

**Resolving Gel Tris-solution**

| Components 4X stock | Concentration per 200 mL |
|---|---|
| 10% SDS | 8 mL |
| Tris base | 36.4 gm |
| pH | 8.8 |
| Distilled water | Up to 200 mL |

**Stacking Gel Tris-solution**

| Components 4X stock | Concentration per 200 mL |
|---|---|
| Tris base | 12.12 gm |
| pH | 6.8 |
| Distilled water | Up to 200 mL |

**10% Ammonium persulphate solution (APS)**

| Components | Concentration |
|---|---|
| Ammonium persulphate | 0.1 gm |
| Distilled water | 1 mL |

**Annexure 2.8:**

**Composition of Western blotting solutions**

**Running buffer**

| Components | Concentration (gm/L) |
|---|---|
| Glycine | 14.4 |
| SDS | 1 |
| Tris-base | 43.2 |

**Transfer buffer**

| Components 20X stock | Concentration per 200 mL |
|---|---|
| Bicine | 16.32 gm |
| Bis-Tris | 20.93 gm |
| Chlorbutanol | 37.3 mg |
| EDTA | 1.2 gm |
| Distilled water | Up to 200 mL |

**10X Tris buffer saline (TBS)**

| Components | g/L |
|---|---|
| NaCl | 88 |
| Tris | 24 |
| pH | 7.6 |
| Distilled Water | Up to 1000mL |

**Wash buffer (TBS-T)**

| Components | Concentration per 100 mL |
|---|---|
| 0.1% Tween-20 | 100 µL |
| TBS buffer | 10 mL of 10X TBS buffer |

**Blocking buffer (TBS-TM)**

| Components | Concentration per 100 mL |
|---|---|
| 0.3% Tween-20 | 100 µL |
| 3% Skim milk powder | 3 gm |
| TBS buffer | 10 mL of 10X TBS buffer |

**Annexure 2.9:**

**ELISA extraction buffer**

| Components | Concentration per 200 mL |
|---|---|
| $Na_2CO_3$ | 15mM |
| $NaHCO_3$ | 35mM |
| $NaN_3$ | 3 mM |
| pH | 9.9 |

# *IN SILICO CHARACTERIZATION OF OUTER MEMBRANE PROTEIN K (OMPK)*

*Chapter 3*

## 3.1. Introduction

In traditional vaccination, there are two types of fish vaccines namely live-attenuated or whole-cell inactivated (WCI) vaccines. WCI vaccination is safe and achieved through inactivation by chemical methods or heat-based inactivation, and these cover the major proportion of aquaculture vaccines employed worldwide (Maiti *et al*., 2020; Munang'andu *et al*., 2014). In contrast, live attenuated vaccines can induce both cell-mediated and humoral immunogenic responses due to their high immunogenicity to counter pathogenic bacteria. But these both types have disadvantages. WCI, less immunogenic, needs multiple doses and sometimes adjuvants to elicit protection for a long time. WCI failed to induce protection against replicating bacterial pathogens such as *A. hydrophila*, *Edwardsiella* spp., and *Piscirickettsia* sp. Whereas, the live-attenuated vaccines can revert to virulence (Munang'andu, 2018; Munang'andu *et al*., 2014). Similarly, other advanced vaccine types also face some problems such as DNA vaccines. Fishes vaccinated through DNA vaccines fall under the category of genetically modified organisms (GMOs) and therefore face GMO regulations (Maiti *et al*., 2020; Munang'andu, 2018). Hence, immunogenic proteins especially outer membrane proteins (OMPs) delivered to fish as genetically engineered vaccines are the best alternative as they induce both humoral and cell-mediated immunogenic responses (Maiti *et al*., 2020).

### 3.1.1. Outer membrane proteins (OMPs)

Outer membrane proteins (OMPs) are a unique and vital component of gram-negative bacteria and prokaryotes. OMPs are also present in specific eukaryotic organelles such as in chloroplast and mitochondria. There have been also some reports of OMPs' presence even in archaea. They play an important role in maintaining the structure of bacteria and help in the transport of materials across the membrane. Most fish pathogens fall into the category of gram-negative bacteria (Achouak *et al*., 2001; Bishop, 2008; Jing *et al*., 2011; Nikaido, 2003; Schulz, 2000). Different studies have revealed that OMPs are potential vaccine candidates, especially against pathogens that infect fish as they serve as protective antigens by stimulating both cellular and humoral responses effectively (Jing *et al*., 2011; Munang'andu, 2018). In gram-negative bacteria, 2-3% of their gene encoding-genome encodes OMPs. OMPs contain β-barrel structures (8-22 β-strands) which display strong tilt on the axis and are arranged in an antiparallel direction to each other (Maiti *et al*., 2020). OMPs in the outer membrane

may be exhibited in the form of monomers, homodimers, and or homotrimers. OMPs' molecular weight ranges from 7-80 kDa. In the OMP structure, the C and N-terminals are oriented towards the periplasm whereas surface loops are exposed to the outer environment as they are located on the exterior of the outer membrane (Jing *et al*., 2011; Maiti *et al*., 2020).

Different studies have revealed that β-sheets strands are more conserved as compared to OMP protein regions near periplasm while in surface loops most variation has been observed in most bacterial species (Schulz, 2000, 2002). The OmpA of *Serratia marcescens* was rich in β-sheet and showed 74% similarity where surface loops and periplasmic turns were around 54%. OmpA helps in the attachment of bacteria to host T-cells (Jing *et al*., 2011; Maiti *et al*., 2020). Similarly, OmpC a porin protein interacts tightly with peptidoglycan and establishes a non-specific channel. Its' variable pore sizes help in metabolic transport, nutrient uptake, and substance synthesis and protect the cell structure by blocking certain antibiotics, detergents, and toxins by acting as a selective barrier (Jing *et al*., 2011; Masi *et al*., 2022). The variations in surface loops account for antigenic diversity within species of bacteria and their exposed location renders them ideal for host immune cell interactions. OMPs enable the pathogens to evade the immune system of the host through anti-complement, anti-phagocytic, and anti-serum bactericidal effects (Jing *et al*., 2011; Koebnik *et al*., 2000).

Outer membrane proteins (OMPs) are regarded as promising candidates for vaccines due to their conserved nature among different serovars and their exposed highly immunogenic epitopes that efficiently promote targeted antibody production. Certain OMPs provided substantial immune protection against respective pathogens ranging from 50-100% (Ebanks *et al*., 2005; Jing *et al*., 2011; Maiti *et al*., 2009, 2012; Neema & Karunasagar, 2018; Vazquez-Juarez *et al*., 2005; Vàzquez-Juárez *et al*., 2004). OMPs have lipopolysaccharides (LPS) that are recognized by the defence cells of a host such as dendritic cells, macrophages, monocytes, and neutrophils. Therefore, OMPs may be important candidates to boost the adaptive immune system to protect for a longer period (Maiti *et al*., 2020).

### 3.1.2. Outer membrane protein K (OmpK)

Outer membrane protein K (OmpK) is a major OMP and a member of the *TSX* protein family. Among *Vibrio* species, it is a common receptor and is associated with regulation

of cell osmotic pressures through a narrow hydrophobic ion channel. Different studies have been conducted which show that *Vibrio anguillarum* isolated OMPs can effectively trigger the immunogenic responses in the understudy organism. NCBI-based OMP gene search has filtered out OmpK, OmpU, OmpW, and TolC as highly conserved proteins through comparative analysis among different *Vibrio* species (Y. Li *et al*., 2010; Lu *et al*., 2014; Mao *et al*., 2011). Research has shown OmpK and OmpW as potent multivalent vaccine candidates against virulent *Vibrio* strains due to high adhesion probability. Investigations into fish vaccines have found OmpK as a promising versatile vaccine candidate and may serve as a diagnostic antigen (Ding *et al*., 2019; Y. Li *et al*., 2010; W. Wang, Guo, *et al*., 2021). OmpK has also been identified as a receptor specifically for the KVP40 phage, which infects at least eight *Vibrio* species (Castillo *et al*., 2019). Xu *et al* (2019b) reported that OmpK as a DNA vaccine can induce both cellular and humoral immunogenic responses in fish. These properties support the use of OmpK as an ideal vaccine candidate to combat the pandemic or endemic-causing pathogenic *Vibrio* species in aquaculture.

### 3.1.3. *In silico* characterization of vaccine candidate

Against infectious diseases, there is an urgent need for potent vaccines (Kardani *et al*., 2020). Vaccines fall under two categories traditional vaccines (inactivated bacteria or live attenuated viruses) and modern vaccines. Traditional vaccines have played a crucial role in saving lives by protecting against diseases like measles, mumps, pertussis, polio, rabies, and smallpox. But these traditional vaccines have some limitations such as reversion to virulence in some cases. To overcome such risks associated with traditional vaccines scientists have started investigating modern vaccine candidates (Jarząb *et al*., 2013; Josefsberg & Buckland, 2012; Pachuk *et al*., 2000; Sunita *et al*., 2020).

Immunoinformatics has emerged as an efficient and powerful way to identify a potent vaccine candidate against a wide range of pathogens. The advancements offered by modern technologies enables scientists to develop multiepitope vaccines that can elicit specific and potent immune responses. Computational vaccinology is emerging as a powerful tool to overcome the challenges associated with designing a vaccine. To investigate immunogenic epitopes in a potent vaccine candidate and to design an effective vaccine from available data, this field uses computational techniques and bioinformatics software and tools (Kardani *et al*., 2020; Meza *et al*., 2017; Moise *et al*.,

2015). One groundbreaking method that is suggested by Dr. Rino Rappuoli is reverse vaccinology for robust modern vaccine development (RV). It involves predicting of potential vaccine candidate from a pathogen's genome by using bioinformatics and computational tools. In RV, the genetic information found in a pathogen's DNA codon sequence is translated into complementary cDNA to yield a desired vaccine candidate (Kardani *et al*., 2020; Z. Yang *et al*., 2021). RV necessitates the identification of immunogenic, non-toxic, and safe substances that can elicit T-cell-mediated and humoral immunity. As a result, modernized web servers for precise immune-dominant component prediction have been established (Baliga *et al*., 2018; Islam, Mahfuj, *et al*., 2022; Parvizpour *et al*., 2020; Sunita *et al*., 2020). Vaccines developed by the RV method offer several advantages in a single recombinant vaccine candidate such as conserved epitope which can elicit immune response and have low risk of reversion to virulence. RV based vaccines also enhances antigen presentation to antigen-presenting cells (APCs) such as dendritic cells which can activate variety of immunogenic cytotoxic T-cells (CTLs) (J.-H. Su *et al*., 2010; W.-Y. Zhou *et al*., 2009). The T-cell epitopes are cleaved proteolytically from the protein and then presented by the MHC molecules that are present on the APCs' surface to interact with the receptor of T-cells (Jensen *et al*., 2018; Jurtz *et al*., 2017).

The development of a vaccine that can elicit effective and strong immunogenic responses is a more intricate challenge to treat and prevent infectious diseases. Many synthetic vaccines may fail due to a limited number of epitopes, inability to mimic the discontinuous epitopes, restricted conserved sequence or population coverage of HLA, and insignificant existence and delivery of epitopes that can elicit T-cell responses. Thus, there is a need to develop a potent and strong vaccines to solve the above mentioned problems by designing and predicting efficient vaccines through the use of modern bioinformatic tools (De Groot *et al*., 2014; Kardani *et al*., 2020; Moise & De Groot, 2006; Moutaftsi *et al*., 2006). Some important points that should be considered before designing a vaccine through RV are briefly described below.

### 3.1.4. Immune systems

There are two main types of immune systems in mammals such as innate and acquired immune systems. Host's first line of defence against pathogens is the innate immune system that is elicited by dendritic cells (DCs) and macrophages. Whereas the adaptive immune system eliminates the pathogen in the later stages of infection (Akira *et al*.,

2006; Kaur *et al*., 2019). The host's innate defence system and inflammatory responses are triggered by the detection of pathogen-associated-molecular patterns (PAMPs) by the host pattern-recognition receptors (PRRs). PAMPs include cell wall components of bacteria and fungus and viral nucleic acid components whereas PRRs encompass AIM-2-like receptors, C-type lectin receptors (CLRs), intracellular nucleic acid sensors (cGAS and OAS protein), leucine-rich repeat-containing receptors (LRRs), nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs), RIG-I-like receptors (RLRs), and toll-like receptors (TLRs) (Kawai & Akira, 2010; Rathinam *et al*., 2012; J. Wu & Chen, 2014). Bacterial pathogens are identified by CLRs (dectin1, dectin-2, and mincle) and TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) whereas bacterial genomic DNA is identified by TLR9 (Gazzinelli *et al*., 2004; Iwasaki & Medzhitov, 2015; Krieg, 2002; Tartey & Takeuchi, 2017).

#### 3.1.4.1. Cell-mediated immunity

Foreign antigen is recognized by T-cells in cell-mediated immunity via endogenous or exogenous antigen process which determines whether the peptide will bind to MHC class I or class II (MHC-I or MHC-II) molecules. Vaccination activates the immune system by targeting cell-mediated immunity. Hence, important point in designing a vaccine candidate is to identify the immunogenic and non-immunogenic epitopes and assess their antigenicity (Dhanda *et al*., 2017; Usmani *et al*., 2018).

#### 3.1.4.2. Humoral immunity

In humoral immunity, the memory B-cells are activated by identifying antigenic regions in pathogenic proteins. These antigenic determines are B-cell epitopes can be potent vaccine candidates against pathogens. It has been proven fact that in eliciting robust humoral response key role is played by T-cells (Dhanda *et al*., 2017; Grimm & Ackerman, 2013; Usmani *et al*., 2018). B-cell epitopes (BCEs) are recognized by B-cell antigen receptors (BCR) which subsequently leads to B-cell differentiation and proliferation into antibody-secreting plasma cells. BCEs have two functional classes linear B-cell epitopes and conformational/distorted epitopes. Linear B-cell epitopes correspond to continuous amino acid residues. Confrontational/distorted epitope sequences are distantly located and elicited upon physical contact with a three-dimensional protein structure (Plotkin, 2010; Usmani *et al*., 2018). Pathogens (influenza, HIV, and malaria) can modify their surface antigens to avoid detection by

the immune system through glycan-masking, mutation, recombination, and variant switching whereas conserved epitopes have been identified recently that can elicit naturally occurring broadly neutralizing antibodies (bnAbs) against these pathogens (Grimm & Ackerman, 2013; Kardani *et al*., 2020).

### 3.1.5. Epitope identification

A crucial step in vaccine design is the identification of immunogenic epitopes. Different studies have shown that immunogenic responses can be multi-specific or broad against pathogens. Immunodominant epitope selection is influenced by several factors such as, 1: The epitope's ability for cellular representation, 2: The epitope's ability to adhere to appropriate MHC molecules, 3: MHC-epitope complex recognition by T-cell repertoire, and 4: competition among T-cells (W. Chen *et al*., 2000; Kardani *et al*., 2020; Sette *et al*., 2002; Sette & Fikes, 2003; van der Most *et al*., 1996; Vitiello *et al*., 1996; Yewdell & Bennink, 1999). The first step in *in silico* vaccine designing involves the bioinformatic analysis of the amino acid sequence of a targeted protein to identify specific motifs which have high affinity towards MHC molecules. There are several HLA molecules' allelic variants, and their frequency varies significantly among diverse ethnic groups. So, therefore it is necessary to compile a set of various epitopes to encompass the global population. Sette *et al*. (2002) proposed that HLA molecules should be grouped as families (HLA supertypes), which have overlapping peptide binding repertoires. This grouping enables researchers to identify peptides that can bind to multiple alleles (Kardani *et al*., 2020).

#### 3.1.5.1. T-cell epitopes prediction

Conventional vaccines based on peptides consist of epitopes that are only identified by T helper (Th) cells or cytotoxic T lymphocytes (CTLs). On the contrary, the original protein within the vaccine contains various HLA-type restricted epitopes that can be identified by both Th cells and CTLs simultaneously. As Th cells play a significant role in eliciting CTLs, therefore the researchers are trying to develop a vaccine that contains both HLA-class I restricted CTL epitopes recognized by CD8+ T-cells and HLA-class II restricted helper cells recognized by CD4+ T-cells. Such a vaccine will have the ability to elicit both Th cells and CTLs. T-cell epitopes through their R-group side chain interact with the groove of MHC-I and II molecules and based on this interaction different computer-based algorithms have been developed by scientists to predict T-

cell epitopes. T-cell epitope prediction can be a direct method or an indirect method (Barve *et al*., 2008; Kardani *et al*., 2020; Kenter *et al*., 2009). The direct method is based on amphipathic patterns epitope motif patterns or a combination of both. The main disadvantage of this method is low accuracy. The indirect method predicts MHC binders rather than T-cell epitopes and is based on 3D-QSAR studies, free energy scoring functions (Fresno), MHC peptide threading, motif patterns, motif profiles, neural networks (ANN), quantitative matrices (QM), and support vector machines (SVM). Due to MHC molecules' binding grooves the prediction of MHC-II binders is more difficult than that of MHC-I because the grooves of MHC-I are closed at both ends whereas MHC-II binding grooves are open at ends (Kardani *et al*., 2020). Different webservers and tools are available to predict MHC-I and II binders as shown in Tables 3.1, 3.2, and 3.3.

#### 3.1.5.2. B-cell epitope prediction

B-cell epitopes, antigenic regions, present on the surface of pathogens are recognized by B-cell receptors resulting in the generation of specific antibodies (Ab). Therefore, B-cell epitopes are suitable candidates for the development of effective vaccines against pathogens. B-cell epitopes can be classified as conformational/discontinuous epitopes or linear epitopes. There are two methods for the prediction of B-cell epitopes due to these grouping based on structures or sequences. Algorithms designed to predict conformational/ discontinuous B-cell epitopes are based on sequence information and 3D structures. In contrast, the algorithms for the prediction of linear B-cell epitopes employ amino acid properties such as charge, hydrophilicity, exposed surface area, and secondary structures (Fleri *et al*., 2017; Soria-Guerra *et al*., 2015). Table 3.4 shows different serves and methods that can be used to predict B-cell epitopes.

### 3.1.6. Allergenicity, antigenicity, and toxicity prediction

A safe and effective vaccine should be non-allergic and non-toxic. More than 500 proteins are classified as allergens in humans by the International Union of Immunological Societies (IUIS). Small proteins which can elicit immunoglobulin E (IgE) antibodies are regarded as allergens. A protein harbouring epitopes that are recognized by both B-cells and Th lymphocytes type-2 (Th-2) can potentially act as an allergen (Cooper, 2004; Furmonaviciene *et al*., 2005). Different bioinformatic tools have been developed to predict the possible allergenicity of a protein or epitopes.

**Table 3.1:** List of web servers for prediction of MHC class I binders

| Server name | Methods of prediction | Coverage |
|---|---|---|
| | **MHC class I Binders** | |
| **EpiJen** | Multi-step algorithm | Predict MHC class I molecules binding to peptide |
| | http://www.ddgpharmfac.net/epijen/ | |
| **IEDB binding** | ANN, CombLib, and SMM-based consensus method | Predict MHC class I molecules binding to peptide |
| | http://tools.immuneepitope.org/mhci/ | |
| **KISS** | Support Vector Machine (SVM) | Predict MHC class I molecules binding to peptides for various arrays of alleles |
| | http://cbio.ensmp.fr/kiss/ | |
| **MMBPred** | Quantitative matrices (QM) | Based on provided protein sequence predicts promiscuous and mutated high-affinity MHC-I binding epitopes |
| | https://webs.iiitd.edu.in/raghava/mmbpred/ | |
| **NetMHCcons** | ANN-based, matrix-based PickPocket, NetMHC, and NetMHCpan in combination | Prediction of peptide binding to MHCI molecule |
| | https://services.healthtech.dtu.dk/service.php?NetMHCcons-1.1 | |
| **NetMHCstab** | Artificial neural network (ANN) | Predicts peptides' binding stability to various MHC molecules |
| | https://services.healthtech.dtu.dk/service.php?NetMHCstab-1.0 | |
| **NetMHCstabpan** | Artificial neural network (ANN) | Predicts peptides' binding stability to MHC molecules |
| | https://services.healthtech.dtu.dk/service.php?NetMHCstabpan | |
| **NetCTLpan** | Artificial neural network (ANN) | Predicts CTL epitopes based on protein sequence provided to MHC molecules |
| | https://services.healthtech.dtu.dk/service.php?NetCTLpan-1.1 | |
| **nHLAPred** | Artificial neural network (ANN) | Predicts MHC-I binding peptides |
| | https://webs.iiitd.edu.in/raghava /nhlapred/ | |
| **ProPred 1** | Matrix-based | Identifies antigen binding regions to MHC-I |
| | https://webs.iiitd.edu.in/raghava/propred1/ | |

**Table 3.2:** List of web servers for prediction of MHC class II binders.

| Server name | Methods of prediction | Coverage |
|---|---|---|
| **MHC class II Binders** | | |
| **Consensus** | Combinatorial library method, SMM-align, and NN-align-based consensus approach | Predicts MHC-II binders |
| | http://tools.immuneepitope.org/mhcii/ | |
| **EpiDOCK** | Docking score-based quantitative matrices (DSQM) | Predicts MHC-II binders |
| | http://www.ddgpharmfacnet/epidock/EpiDockPage.html | |
| **EpiTOP** | Quantitative matrices (QM) | Predicts MHC-II binding |
| | http://www.ddgpharmfac.net/EpiTOP3/ | |
| **IEDB binding** | CombLib, SMMalign, Sturniolo, and NN-align combined approach | Predicts peptide binding to MHC-II molecule |
| | http://tools.immuneepitope.org/mhcii/ | |
| **IMTECH** | Quantitative matrices (QM) | Predicts binding core of MHC-II |
| | http://crdd.osdd.net/raghava/mhc/ | |
| **HLA-DR4Pred** | Artificial neural network (ANN) and Support vector machine (SVM) | Predicts HLA-DRB1*0401 binders in antigen sequence |
| | https://webs.iiitd.edu.in/raghava/hladr4pred/ | |
| **ProPred** | Quantitative matrices (QM) | Predicts peptide binding regions in MHC-II molecule |
| | https://webs.iiitd.edu.in/raghava/propred/ | |

**Table 3.3:** List of web servers for prediction of MHC class I and II binders

| Server name | Methods of prediction | Coverage |
|---|---|---|
| **MHC class I and II Binders** | | |
| **EpiMatrix** | Matrix-based and pocket profile-based algorithm | Predicts MHC class I and II binding peptides |
| | http://www.epivax.com/ | |
| **MHCPred** | Additive method | Predicts MHC class I and II molecules' binding affinity |
| | http://www.ddgpharmfac.net/mhcpred/MHCPred/ | |
| **MULTIPRED 2** | MULTPRED, NetMHCpan, and PEPVAC based algorithm | Predicts binding affinity of peptides to multiple alleles of HLA* class I & II DR molecules |
| | http://cvc.dfci.harvard.edu/multipred2/index.php | |

* HLA: Human leukocyte antigen

**Table 3.4:** List of web servers for prediction of B-cell epitopes based on structures and sequences

| Server name | Methods of prediction | Coverage |
|---|---|---|
| **Based on structure** | | |
| **CBTOPE** | Support vector machine (SVM) | Predicts antigen sequence-based discontinuous B-cell epitopes |
| | https://webs.iiitd.edu.in/raghava/cbtope/ | |
| **DiscoTope2.0** | Based on exposure of half-sphere and propensity scores | Predicts antigenic structure-based conformational B-cell epitopes |
| | https://services.healthtech.dtu.dk/service.php?DiscoTope-2.0 | |
| **ElliPro** | A residue clustering algorithm and Thornton's method | Predicts B-cell epitopes (discontinuous and linear) |
| | http://tools.immuneepitope.org/ellipro/ | |
| **LYRA** | BLOSUM matrix and Homology-based model | Predicts B-cell and T-cell receptors' structure |
| | https://services.healthtech.dtu.dk/service.php?LYRA-1.0 | |
| **PEASE** | Antibody sequence algorithm | Predicts B-cell epitope specific to antibody |
| | www.ofranlab.org/PEASE | |
| **Based on sequence** | | |
| **ABCPred** | Artificial neural network (ANN) | Predicts antigen sequence-based B-cell epitopes |
| | https://webs.iiitd.edu.in/raghava/abcpred/ | |
| **BepiPred 2.0** | Random forest model | Predicts antigen sequence-based B-cell epitopes |
| | https://services.healthtech.dtu.dk/service.php?BepiPred-2.0 | |
| **BCPREDS** | String kernels-based and support vector machine-based algorithm | Predicts B-cell epitopes of flexible length |
| | http://ailab.ist.psu.edu/bcpred/ | |
| **COBEPro** | Support vector machine (SVM) | Predicts continuous B-cell epitopes |
| | http://scratch.proteomics.ics.uci.edu/ | |
| **LbTope** | K-nearest neighbour and support vector machine algorithm | Predicts linear B-cell epitopes |
| | https://webs.iiitd.edu.in/raghava/lbtope/ | |

These tools are either based on a sequence similarity search model or based on a conserved allergenicity-linear motif identification model. A set of amino acids responsible for specific activity is known as a motif (Dimitrov *et al*., 2014; Fiers *et al*., 2004; Ivanciuc *et al*., 2003; Stadler & Stadler, 2003). A bioinformatic tool AllerTOP ver. 2 by comparing the fingerprint of a target protein and the fingerprint of a set of known 2427 allergens, predicts the allergenicity according to the Tanimoto index in the target protein. This model has 87% efficiency in predicting the allergenicity among the test protein (Kardani *et al*., 2020; Venkatarajan & Braun, 2001).

The effective vaccine must be immunogenic which refers to its ability to elicit both cellular and humoral immunogenic responses which results in memory cell generation against pathogenic epitopes. The determining of immunogenicity is a critical step as most of the peptide-based vaccine's major disadvantage is their low immunogenicity. Several web servers are available to predict the antigenicity and immunogenicity of target proteins such as the VaxiJen web server. This server is used to predict the immunogens, protective antigens, subunit vaccines, and tumour antigens in bacteria, fungi, parasites, tumours, and viruses (M. Ali *et al*., 2017; Doytchinova & Flower, 2007, 2008; Rappuoli *et al*., 2002).

To predict the toxicity of peptides very limited efforts have been made to develop any bioinformatic tools. Chaudhry and colleagues recently created a repository of experimentally validated hemolytic peptides from various sources to develop different models to predict hemotoxicity. These models used various features of peptides such as binary profiles, hemolytic motifs, and residue composition. Another important factor is the autoimmune reaction of test protein before considering it as a vaccine candidate (K. Chaudhary *et al*., 2016; Gautam *et al*., 2014; Kanduc & Shoenfeld, 2019; Raghava *et al*., 1994). Table 3.5 shows some web servers used for the prediction of allergenicity, toxicity, and antigenicity.

### 3.1.7. Physiological parameters evaluation

Human insulin was the first recombinant therapeutic protein which opened the doors for recombinant proteins as a new class of therapeutic products that has been widely used in treating cancer, diabetes, and other infectious diseases and disorders (Goeddel *et al*., 1979; J Boohaker *et al*., 2012; Leader *et al*., 2008; Vlieghe *et al*., 2010).

**Table 3.5:** List of web servers for prediction of allergenicity, toxicity, and antigenicity

| Server name | Methods of prediction | Coverage |
|---|---|---|
| | **Allergenicity** | |
| **AllergenFP** | Fingerprint algorithm based on alignment-free descriptor | Identifies non-allergens and allergens |
| | http://ddgpharmfac.net/AllergenFP | |
| **AllergenPro** | FAO/WHO, epitope-based and motif-based methods | Predicts well-known allergens' potential cross-reactivity |
| | http://nabic.rda.go.kr/allergen/ | |
| **AllergenOnline** | FAO/WHO parameters | Provides a database for allergens that helps in the recognition of potential allergenic proteins |
| | http://allergenonline.org/ | |
| **Allermatch** | FAO/WHO parameters | Predicts potential allergenic proteins |
| | http://allermatch.org/ | |
| **AllerTOP 2.0** | Auto cross-covariance | Predicts allergenicity |
| | http://www.ddgpharmfac.net/AllerTOP/ | |
| **AlgPred** | ARPs BLAST, IgE epitope, MAST, and SVMc methods | Predicts allergenic proteins and maps IgE epitopes |
| | https://webs.iiitd.edu.in/raghava/algpred/ | |
| **SDAP** | Combined with additional bioinformatics servers | Provides a database of allergenic proteins based on structure |
| | http://fermi.utmb.edu/SDAP/ | |
| | **Toxicity** | |
| **ToxinPred** | Based on position-specific scores and quantitative matrix | Predicts non-toxic/toxic peptides |
| | https://webs.iiitd.edu.in/raghava/toxinpred/ | |
| | **Antigenicity** | |
| **ANTIGENpro** | Alignment-free, pathogen-independent, and sequence-based methods | Predicts antigenicity of the protein |
| | http://scratch.proteomics.ics.uci.edu/ | |
| **VaxiJen** | Alignment-based | Predicts subunit vaccines and protective antigens |
| | http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html | |

These therapeutic peptides or proteins were highly versatile, specific, and less toxic but low solubility, non-immunogenicity, proteolytic degradation, physiochemical instability, and short half-life are some disadvantages that have been observed (Antosova *et al*., 2009; Bruno *et al*., 2013; Di, 2015; Fosgerau & Hoffmann, 2015; Vlieghe *et al*., 2010). The web server THPdb (https://webs.iiitd.edu.in/raghava/thpdb/) is a repository site that contains administration route, chemical properties, composition, disease area, mode of activity, physical appearance, pharmacological class, pharmacodynamics, sequence, structure, the target of activity, and toxicity of each protein/peptide-based therapeutics and their corresponding drug variants which are approved by FDA (Usmani *et al*., 2017). The main challenge is to determine the stability of a novel vaccine candidate in blood via its half-life in blood. *The in silico* approach to predict to half-life of peptides is a valuable tool for researchers. The *in silico* tools such as ProtLifePred (http://protein-n-end-rule.leadhoster.com/) and Protparam (http://web.expasy.org/Protparam) are used to determine the half-life of vaccine candidates based on the N-end rule in *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) and mammalian cells. Other physiochemical properties such as aliphatic index, extinction coefficient, grand average of the hydrophobicity (GRAVY) value, instability, molecular weight (MW), solubility, and theoretical isoelectric pH (pI) can also be determined by ExPASy (http://web.expasy.org/Protparam) web server (Artimo *et al*., 2012; Gasteiger *et al*., 2005; Khalid *et al*., 2022; Magnan *et al*., 2009). The vaccine candidate solubility can be cross-checked by the SOLpro (http://scratch.proteomics.ics.uci.edu) web tool. The accuracy of this tool is approximately 74% (Cheng *et al*., 2005; León *et al*., 2020; Magnan *et al*., 2009).

### 3.1.8. Protein structure prediction

The protein's secondary and tertiary structures are crucial in understanding their function and interactions within a biological system. The secondary structure such as alpha helices, beta sheets, and coils provides information about the local folding patterns of proteins. These local folding patterns help in understanding the essential regions of proteins such as domains, motifs, and post-translation modification sites that may be involved in specific interactions and functions (Guharoy & Chakrabarti, 2007; B. Jiang *et al*., 1998; Kuhar *et al*., 2021; Offmann *et al*., 2007; Planat *et al*., 2021). Two-dimensional (2D) model prediction tools have been used by researchers to predict

protein functional regions which aid them in designing the vaccine candidate with interpreted results. Such models also help them to annotate the protein sequences and their structural features along with function predictions. Such models also help the researchers to study the evolutionary history of proteins and the mutation impact on their function (Agamah *et al*., 2020; Engelhardt *et al*., 2005; Islam, Mou, *et al*., 2022; Marks *et al*., 2012; Reva *et al*., 2011; Studer *et al*., 2013; Whisstock & Lesk, 2003; Yu *et al*., 2022). The tertiary structure of the protein provides overall information about the structure and folding of the protein. The tertiary structure is crucial in understanding the functional mechanisms of the protein such as the binding of ligands, catalysis of enzymes, and peptide-peptide interaction and helps in decapitating the drug design and discovery (Eisenhaber *et al*., 1995; Kristiansen, 2004; Liljefors *et al*., 2002; Marín-López *et al*., 2018; Meiler & Baker, 2003; Milroy *et al*., 2014; Norouzi *et al*., 2022; Rehman *et al*., 2017).

Researchers use three-dimensional (3D) model predicting tools to study drug binding sites, design novel therapeutics, establish links between protein structure and function, and study molecular dynamics, and flexibility of protein. Such tools also help them understand the impact of mutations on the interaction of drugs with the targeted protein, and interactions of proteins with each other. Understanding of interacting proteins spatial arrangements helps in elucidating the cellular processes, disease mechanisms, and signaling pathways. The 3D arrangement of amino acids and how specific motifs or domains contribute to the biological activity of the protein. The 3D simulation of proteins explains conformational changes, interactions over time, and stability of proteins and enables the researchers to understand the behaviour of proteins (Hernández-Rodríguez *et al*., 2016; Iglesias *et al*., 2018; Isert *et al*., 2023; Kuhlman & Bradley, 2019; X. Liu *et al*., 2018; Schmidt *et al*., 2014; K. Wu *et al*., 2023).

In short, the 2D model of the proteins provides information about the structural features and sequence characteristics of proteins, while the 3D model offers a holistic view of the overall structure to understand its functions and interactions (Chakrabarty & Parekh, 2016; Cheng *et al*., 2005; Robert & Gouet, 2014). In 1984, the first attempt was made to predict the 3D structure of a protein based on epitope. The most successful and effective method to predict the structure of the protein is through the determination of homologs whose tertiary structure is already predicted, this model is cited as fold recognition/ template-based homology modelling (Jisna & Jayaraj, 2021; Ponomarenko

*et al*., 2008; D. Xu *et al*., 2000). The list of web servers used to predict secondary and tertiary structures of proteins is given in Table 3.6.

**Table 3.6:** List of web servers for prediction of secondary and tertiary structures

| Server name | Prediction method | Coverage |
|---|---|---|
| **Secondary structure** | | |
| **PSIPRED** | Machine learning and position-specific scoring matrices | Predicts the secondary structure (α-helices, β-sheets, & coils) |
| | http://bioinf.cs.ucl.ac.uk/psipred | |
| **DSSP** | Hydrogen bonding patterns | Predicts the secondary structure (helix, sheet, coil) to each amino acid residue in a protein |
| | https://swift.cmbi.umcn.nl/gv/dssp/ | |
| **SOPMA** | Self-optimized method | Predicts amino acids for a three-state description of the secondary structure (α -helix, β -sheet, and coil) |
| | http://www.prabi.fr/ | |
| **Tertiary structure** | | |
| **GalaxyRefine** | Performs repeated structure perturbation followed by overall structural relaxation by molecular dynamics simulation. | Predicts and refines protein structure |
| | https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE | |
| **I-TASSER** | ab initio modeling, structural refinement, and template-based modeling in combination | Predicts the 3D structure of a protein and provides confidence scores |
| | https://zhanglab.ccmb.med.umich.edu/I-TASSER/ | |
| **Raptor X** | Multiple threading programs and template-based modeling | Predicts 3D structures, disorder regions, and solvent accessibility |
| | http://raptorx.uchicago.edu/ | |
| **Robetta** | ab initio methods and homology modeling | Generate 3D models for the given protein sequence |
| | http://robetta.bakerlab.org/ | |
| **SWISS-Model** | Homology modeling. | Predict the structure of a target protein based on templates from known structures |
| | http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html | |
| **Phyre** | Homology modeling | Predicts protein 3D structures |
| | http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index | |

### 3.1.9. Protein-peptide docking

Molecular docking is another way to design an effective vaccine (Diller *et al*., 2015; Schueler-Furman *et al*., 2017). In computational docking, the peptide-protein interaction and binding site of a receptor and ligand are predicted through different algorithms. The algorithms that are currently in use are global docking, local docking, and template-based docking to predict peptide-protein interactions. Global docking is based on the combined search for the binding site/state of a peptide and reads the input conformational structure of protein and peptide as rigid and then performs a rigid-body docking (Porter *et al*., 2017; C. Yan *et al*., 2016). The local docking model searches the peptide binding site according to user-defined binding site information. The accuracy of this model relies on the information provided by a user for better output. In contrast to the above-mentioned model template-based docking uses known template structures as platforms to predict peptide-protein interactions (Antes, 2010; Kundrotas *et al*., 2012; H. Lee *et al*., 2015; Lensink *et al*., 2017; London *et al*., 2011). Some of the servers that are used for computational docking are mentioned in Table 3.7.

**Table 3.7:** List of web servers for molecular docking

| Server name | Prediction methods | Coverage |
|---|---|---|
| | **Molecular docking servers** | |
| **ClusPro** | Based on rigid body docking, root-mean-square deviation (RMSD), and refinement of selected structures | Dock two interacting proteins |
| | https://peptidock.cluspro.org/ | |
| **GalaxyPepDock** | Based on interaction similarity | Predicts protein-peptide docking |
| | http://galaxy.seoklab.org/index.html | |
| **MDockPeP** | Based on modeling peptide conformers, protein-peptide binding modes and scores | Predicts protein-peptide complex structures |
| | http://zougrouptoolkit.missouri.edu/mdockpep/ | |
| **PatchDock** | Molecular docking algorithm based on shape complementarity principles | Dock two interacting proteins |
| | http://bioinfo3d.cs.tau.ac.il/PatchDock/ | |
| **ZDOCK** | Fast Fourier Transform (FFT) based protein docking program | Dock two interacting proteins |
| | http://zdock.umassmed.edu | |

### 3.1.10. Codon Optimization

Numerous recombinant proteins have been produced through biotechnology successfully and this recombinant technology facilitated the advancement in diagnostics, medicine, therapeutics, and scientific research that was previously challenging to achieve (Haridhasapavalan *et al*., 2020; Nezafat *et al*., 2015). These recombinant proteins' heterologous production faces different challenges. The most prominent one is codon usage bias which may affect the protein expression efficiency and fidelity when expressed in a heterologous system (Dey, C., Narayan, G., Krishna Kumar, H., Borgohain, M. P., Lenka, N., & Thummer, 2016; Haridhasapavalan *et al*., 2020). This problem can be addressed through codon optimization by researchers to express the protein of interest in specific expression hosts (Burgess-Brown *et al*., 2008; Maertens *et al*., 2010). For expression in *Escherichia coli*, the codon optimization can be achieved by Thermofisher Scientific GeneOptimizer (https://www.thermofisher.com/in/en/home/lifescience/cloning/genesynthesis/geneart-gene-synthesis/geneoptimizer.html) (Haridhasapavalan *et al*., 2020). The GenSmart™ Codon Optimization (https://www.genscript.com/gensmart-free-gene-codon-optimization.html) is another online tool to accomplish codon optimization. Codon optimization performed by GenSmart™ Codon Optimization web servers can also determine whether a target gene is being expressed in the desired expression system by analysing the GC content of the understudy sequence. In this regard having a GC content of between 30 and 70% indicates optimum expression (Cheema *et al*., 2022; Gieras *et al*., 2008; Goulet *et al*., 2022; Jain *et al*., 2023; Y. Yao *et al*., 2023).

### 3.1.11. Objectives

In the current part of the study, the objective was to design the full-length OmpK vaccine for the prevention of vibriosis in fish. To assess the biological properties such as MHC-I and II binding epitopes, B-cell epitopes, toxicity, antigenicity, allergenicity, and other physiochemical parameters different bioinformatic tools were used. The secondary and tertiary structure of the vaccine construct was also generated with the use of different web servers followed by refinement and validation. Then molecular docking was performed to assess the interaction of the vaccine construct with innate immune receptors (Toll-like receptors (TLRs)) to check the immunogenicity of the vaccine construct.

## 3.2. Materials and Methods

### 3.2.1. Retrieval of antigen sequence and proteome retrieval

The outer membrane protein K (OmpK) gene sequence (Gene bank accession no. **FJ705222.1**) was retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and this sequence was translated into the amino acid sequences by web server "EMBOSS Transeq Tool" (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). Basic Local Alignment Search Tool for Protein (BLASTp) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), analysis was performed to determine the level of conservation of the protein among different *Vibrio* strains.

### 3.2.2. Sequence modification

The OmpK sequence was modified by the addition of six histidine amino acid residues (6xHis) at the C terminal of the OmpK protein sequence to be used as an OmpK vaccine construct (OVC). The 6xHis was added for detection via Western blotting and ELISA after transformation.

### 3.2.3. Epitope Prediction

Epitopes are usually short peptides that can be constructed from the pathogen's antigenic proteins and can interact with the immune receptors of the host to cause an immune response (Malito *et al*., 2013; Mikala & Vályi-Nagy, 2002). Different online tools can be used to predict the immunodominant epitopes that would activate Cytotoxic T-cells (CTLs), Helper T-cells (HTLs), and B-cells. In all the servers used, the OVC sequence was uploaded in FASTA format.

#### 3.2.3.1. Prediction of MHC-I binding epitopes

Activation of CTLs requires antigenic epitopes that are bound to MHC-I are very important. The recommended web server Immune Epitope Database Nathan EL 4.1 (http://tools.immuneepitope.org/mhci/) was used to find the most immunodominant MHC-I binding CTLs epitopes in the OVC. The IEDB MHC-I binding prediction tool is a reliable way to predict MHC-I binding epitopes. It is a freely accessible online tool that has vast applications in the field of bioinformatics and biomedical research (Fleri *et al*., 2017). The default HLA allele reference set was chosen based on the most

common MHC alleles. The FASTA format was then submitted to the MHC-I epitope mapping group.

#### 3.2.3.2. Prediction of MHC-II binding epitopes

Helper T-cells (HTLs) are the most important part of adaptive immunity because they help activate CTLs and B-cells so they can make antibodies. So, the immunodominant epitopes are against HTLs that can bind to MHC-II and turn on HTLs when the vaccine is introduced (Alberts *et al*., 2002; Samad *et al*., 2022). So, the *in silico* analysis of the target OVC for MHC-II binding epitopes was carried out through the Immune Epitope Database (IEDB) MHC-II (http://tools.iedb.org/mhcii/). It measured the affinity of binding between MHC-II and epitopes in terms of $IC_{50}$. All the parameters were set to their default values, which were: prediction method IEDB 2.22, HLA-DR, selected length fifteen for OVC, and full HLA reference set for epitope mapping. The values < 50 nM refer to strong, < 500 nM refer to moderate, and < 5000 refer to weak binding affinities of epitopes for MHC-II.

#### 3.2.3.3. Prediction of B-cell epitopes

Vaccines must activate B-cells to make a strong antibody response. Linear B-cell epitopes were predicted using the online web server ABCpred (http://crdd.osdd.net/raghava/abpred/). This server is based on an artificial neural network (ANN) and recurrent neural network to predict B-cell epitopes with 65.93% accuracy (Saha & Raghava, 2006, 2007). Additionally, the server provides users with the flexibility to choose the length of the window from a range of window length options of 10, 12, 14, 16, and 20. The window length of 16 and the threshold of 0.8 was set. The overlapping filter was turned off.

#### 3.2.3.4. Antigenicity, allergenicity, and toxicity analysis of predicted epitopes

All the epitopes were analyzed for antigenicity, allergenicity, and toxicity. For this purpose, different online tools were used. For the prediction of epitopes' protective antigenicity VaxiJen v2.0 (https://mybiosoftware.com/vaxijen-2-0-prediction-of-protective-antigens-and-subunit_vaccines.html/), for allergenicity AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/), and for toxicity ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) was used. VaxiJen classifies antigens by converting protein into a set of essential amino acid components based on Automated Cross-Covariance (ACC), an innovative treatment-agnostic method that overcomes the

limitations of the joint method (Doytchinova & Flower, 2007). The AllerTOP v. 2.0 method predicts recombinant protein allergenicity based on autocross covariance ACC, which describes residue hydrophobicity, size, abundance, helicity, and β-strand formation propensity. Compared to many allergy prediction methods, AllerTOPv.2 has the highest accuracy (88.7%) (Dimitrov *et al*., 2014). ToxinPred is an efficient online tool that enables the prediction of the toxic potential of peptides, and the screening of non-toxic peptides (Gupta *et al*., 2013; Rathore *et al*., 2023; N. Sharma *et al*., 2022). In this study, all the predicted CTL, HTL, and B-cell epitopes were submitted in FASTA format in the 'Batch Submission' option of ToxinPred to determine toxicity. Moreover, amongst the SVM and Quantitative Matrix (QM) methods, the 'SVM (Swiss-Prot) based' method was selected. Along with this, other parameters were set at default including the E-value cut-off for the motif-based method>10 and the SVM threshold>0.0.

### 3.2.4. Characteristics evaluation of OmpK vaccine construct

Modified amino acid sequence of OmpK, which has six histidine residues at the C terminal of amino acids was used as an OmpK vaccine construct (OVC). The different physiochemical properties of the OmpK vaccine construct as well as the antigenicity, allergenicity, and toxicity analysis of the modified OmpK vaccine construct were checked using different tools. Toxicity, allergenicity, and antigenicity analysis were done for the OVC sequence, using online tools VaxiJen v2.0 (https://mybiosoftware.com/vaxijen-2-0-prediction-of-protective-antigens-and-subunit vaccines.html/), AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/), and ToxinPred (http://crdd.osdd.net/raghava/toxinpred/), respectively. The physicochemical properties of the OVC such as amino acid composition, molecular weight, theoretical isoelectric point (pI), atomic composition, expected half-life *in vitro* and *in vivo*, grand average of the hydropathicity (GRAVY) value, extinction coefficient, instability index (II), and aliphatic index of the vaccine construct was assessed with the help of the tool ExPASy Protparam (https://web.expasy.org/protparam/) (Artimo *et al*., 2012; Gasteiger *et al*., 2005). The SOLpro of the SCRATCH Protein Predictor tool (http://scratch.proteomics.ics.uci.edu/) was used to find out the extent of the solubility of the vaccine construct upon its expression.

### 3.2.5. Two and three-dimensional structures of OVC

Structural analysis is an important step during *in silico* vaccine designing because, in *in vivo* conditions, the structure of proteins critically influences their functioning. Hence, the 2D and 3D structural analysis was performed for both vaccine constructs. The 3D structure generation was followed by refinement and validation to obtain a better-quality 3D structure.

#### 3.2.5.1. Two-dimensional structure prediction

SOPMA achieves significant protein secondary structure prediction by estimating the similarity from multiple alignments (Abubaker, 2015). The secondary structure of the vaccine construct was found by the SOPMA server (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html). The OVC amino acid sequence in FASTA format was inserted and default settings were selected to obtain secondary structure.

#### 3.2.5.2. Three-dimensional structure generation and refinement

To generate and refine the 3D structures of the vaccine constructs, the GalaxyRefine tool (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) was used. Galaxy generates and refines the 3D models of proteins based on molecular dynamics simulation. This method showed one of the best performances in improving protein structure quality (Enayatkhani *et al*., 2021; Heo *et al*., 2013). The tertiary structure of vaccine constructs must be predicted during *in silico* analysis because the structure of proteins affects how they function.

#### 3.2.5.3. Three-dimensional structure validation

After the completion of model generation and refinement, it was essential to investigate the quality of the generated models. Hence, the resulting models for OVC obtained from GalaxyRefine were validated by a series of tools for predicting their quality. Two online bioinformatics tools were used for model quality validation, namely Saves v6.0 server (https://saves.mbi.ucla.edu/) and Protein Structure Analysis (ProSAweb) (https://prosa.services.came.sbg.ac.at/prosa.php) tool.

The Saves v6.0 server provides a comprehensive analysis of quality validation through programs including ERRAT, Verify3D, PROVE, WHATCHECK, and PROCHECK. However, for the quality evaluation of OVC 3D models generated in this study were verified using the ERRAT and PROCHECK programs as followed by Sharma *et al.*

(2021). The ERRAT program determined the quality factors of the models, and the best models were picked based on the highest quality factors predicted (Lovell *et al*., 2003; Obukhov, 2002). However, the PROCHECK program was used for the construction of Ramachandran plots for the models. Ramachandran plots were used to investigate the number of residues in the allowed and disallowed regions. The models with a greater number of residues in the disallowed regions of the Ramachandran plot had poor model quality (Laskowski *et al*., 2006). Hence, the PDB files of the models were provided as input to this tool, and the ERRAT and PROCHECK programs were performed.

ProSAweb was another online tool used for model quality evaluation and validation. This webserver enables model validation by predicting the Z-score of the model and generating the energy plot. A low Z-score indicates better quality of the model; similarly, the energy plot reflects model quality by demonstrating the energy considerations of the model. However, in this analysis, the Z-score determination was performed to evaluate the model quality (Wiederstein & Sippl, 2007). For this purpose, the Protein Data Bank (PDB) files of the models for OVC were provided to the software, and analysis was made based on the Z-score. Finally, the validation of refined models enabled the selection of refined models of the highest quality for both vaccine constructs that were further used for downstream analysis.

PyMOL (https://pymol.org/) is a freely accessible cross-platform-based molecular structure visualization system that enables visualization of 3D structures at the molecular level. Along with visualization, it offers multiple other functions as well including model editing (DeLano, 2002; Yuan *et al*., 2017). So, the 3D structures of OVC were visualized by opening their PDB files in the PyMOL software.

### 3.2.6. Discontinuous B-cell Epitope Mapping

Discontinuous or conformational B-cell epitopes on the surface of antigen get recognized by the B-cell receptors leading to the generation of immune response by inducing antibody production (Kardani *et al*., 2020). ElliPro is a program of the IEDB tool that allows rapid computation of linear as well as conformational B-cell epitopes. Therefore, conformational B-cell epitopes were mapped on the vaccine candidates using ElliPro (http://tools.iedb.org/ellipro/) webserver. In the computation of the conformational B-cell epitopes, the default parameters were used including the ‘Maximum score’ of 0.5 and the ‘Maximum distance’ of 16. PDB files of the refined

model of the designed vaccine construct were submitted to the ElliPro server for discontinuous epitope computation.

### 3.2.7. Molecular Docking

To investigate how vaccine constructs interact with immune cell receptors, molecular docking was carried out. ClusPro 2.0 Protein-Protein Docking Server (https://cluspro.bu.edu/publications.php) was used for this purpose. It was an open-access online tool. This server performs rigid body docking by implementing techniques of correlation using the Fast Fourier Transform (FFT) and generates several ligand-receptor interactions based on the lowest binding energy values. The users can pick the ligand-receptor complex based on least Lowest Binding Energy. Regarding this aspect, the Toll-Like Receptor 5 (TLR5) of immune cells was utilized as a receptor, and the designed vaccine construct was docked with it. The PDB file of TLR-5 was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (https://www.rcsb.org/) (RCSB PDB ID: 3J0A) (A. Joshi *et al.*, 2021). The TLR-5 complex PDB file had two chains (chain A and chain B). Consequently, to facilitate efficient docking between the TLR-5 and OVC we only selected chain A. Chain B was eliminated through the PyMOL software. This chain B free TLR-5 PDB file was added as the receptor whereas the OVC was added as the ligands. The rest of the parameters were set to default and the docking analysis was performed.

### 3.2.8. Codon Optimization

It is necessary to introduce the potential vaccines into the host system to cause the expression of the vaccine candidates. The web server GenSmart™ Codon Optimization (https://www.genscript.com/gensmart-free-gene-codon-optimization.html) was used to perform codon optimization on the OVC construct per plant codons to facilitate its expression. Codon optimization of the vaccine design was done for the plant *Nicotiana tabacum*. GenSmart™ Codon Optimization web servers also determine whether a target gene is being expressed in the host system or not by analyzing the GC content of the codon. In this sense, having a GC content of between 30 and 70% indicates optimum expression (Sanami *et al.*, 2021). CLUSTAL O (1.2.4) multiple sequence alignment (https://en.vectorbuilder.com/tool/sequence-alignment.html) was used for the

alignment of DNA and translated sequences of both NCBI retrieved sequence and codon-optimized sequences on the web server.

## 3.3. Results

### 3.3.1. Antigen sequence retrieval and OmpK vaccine construct

From NCBI the *OmpK* gene (Gene bank accession no. FJ705222.1) was retrieved as shown in Figure 3.1 (A). The six histidine residues at the C-terminal of the NCBI retrieved sequence were added for detection via Western blotting and ELISA in the later stage of the experiment. After modification, the translation of gene sequence to amino acid sequence was performed by the EMBOSS Transeq Tool. A total of 269 amino acids were found in the final OVC. The BLASTp results showed the conserved nature of the OmpK protein with the conserved domain database ID 225787 belonging to the *Tsx* protein family. The whole translated sequence is shown in Figure 3.1 (B).

### 3.3.2. Epitopes prediction

For predicting immune-dominant and safe epitopes for activation of vital immune system cells such as CTLs, HTLs, and B-cells, the OVC sequence was examined for MHC-I, MHC-II binding epitopes, and B-cell epitopes. MHC-II binding epitopes are crucial for the activation of Helper T-cells. OVC sequence was provided to the IEDB MHC-I tool, ABCpred, and IEDB MHC-II tool for prediction of MHC-I binding epitopes, B-cell epitopes, and MHC-II binding epitopes, respectively, as an output total of ten MHC-I binding epitopes (Table 3.8), five MHC-II binding epitopes (Table 3.9), and five B-cell epitopes (Table 3.10) were identified. Hence, the final, antigenic, non-allergenic, and non-toxic MHC-I, B-cells, and MHC-II binding epitopes and the length of epitopes, the start/end numbers of amino acids of each epitope are shown in respective tables in detail.

>FJ705222.1 *Listonella anguillarum* strain NB10 outer membrane protein K (ompK) gene, complete cds

ATGCGTAAATCACTTTTAGCTCTAGGCCTAGTGGCTGCAACTTCTGCTCCTGTTATG
GCCGCTGACTATTCAGATGGCGACATCCATAAAAACGATTACAAATGGATGCAATTT
AACCTAATGGCTGCAATCGATGAATTACCAGGTGAATCATCGCATGATTACCTAGAG
ATGGAATTTGGCGGCCGCTCAGGGATTTTTGATCTGTACGGTTACGTTGATATCTTT
AACCTGCTAAGCAACCCAAGCAGTGACAAAGAAGGTAAAGAAAAAATCTTTATGAAA
TTTGCACCTCGTATGTCACTAGATGCACTTACAGGTAAAGATTTGTCTTTCGGGCCA
GTGCAAGAGTTGTATGTCTCTACGCTAATGGAATGGGGTGGTAACTCTGGCGTTAAC
ACTCAAAAAGTGGGCCTTGGCTCTGATGTGAATGTGCCTTGGTTTGGTAAAGTGGGT
TTAAACCTTTACGGTACTTATGATTCAAATGAGAAAGATTGGAACGGCTTCCAAATT
TCAACCAACTGGTTCAAACCTTTTTACTTCTTTGAAAACGGCTCGTTTATCTCTTAT
CAAGGTTACATCGATTACCAATTTGGCATGGATGACAAAAATACTGCACTAAAAACC
TCAAATGGTGGTGCAATGTTTAACGGTATTTACTGGCACTCCGATCGCTTTGCTGTT
GGCTACGGCCTTAAAGGCTACAAAGATGTTTATGGTCTGAAAGATGAAGGTCTTGCA
GGCAAAACAACTGGCTTTGGTCACTACCTAGCAGTAACTTACAAGTTCTAA

**(A)**

> Outer membrane protein K (ompK)with 6xHis tag

MRKSLLALGLVAATSAPVMAADYSDGDIHKNDYKWMQFNLMAAIDELPGESSHDYLE
MEFGGRSGIFDLYGYVDIFNLLSNPSSDKEGKEKIFMKFAPRMSLDALTGKDLSFGP
VQELYVSTLMEWGGNSGVNTQKVGLGSDVNVPWFGKVGLNLYGTYDSNEKDWNGFQI
STNWFKPFYFFENGSFISYQGYIDYQFGMDDKNTALKTSNGGAMFNGIYWHSDRFAV
GYGLKGYKDVYGLKDEGLAGKTTGFGHYLAVTYKFHHHHHH

**(B)**

**Figure 3.1**: **Schematic demonstration of the OmpK DNA and protein sequence.** **(A)** NCBI retrieved non-modified sequence. **(B)** OmpK vaccine construct (OVC) and its primary translated protein sequence. Histidine residues (Red) were added at the C-terminal of the OmpK sequence (Blue) to modify it.

**Table 3.8:** Predicted MHC-I binding epitopes and their antigenicity, toxicity, and allergenicity.

| **Allele** | HLA A* 23:01 | HLA B* 07:02 | HLA A* 24:02 | HLA A* 23:01 | HLA A* 31:01 | HLA B* 15:01 | HLA B* 58:01 | HLA A* 03:01 | HLA B* 40:01 | HLA B* 07:02 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Epitopes binding Predictions** | | | | | | | | | | |
| **Start** | 255 | 97 | 189 | 174 | 91 | 231 | 139 | 227 | 58 | 96 |
| **End** | 263 | 105 | 198 | 182 | 99 | 239 | 147 | 236 | 66 | 105 |
| **Length** | 9 | 9 | 10 | 9 | 9 | 9 | 9 | 10 | 9 | 10 |
| **Epitope Sequence** | HYLAVTYKF | APRMSLDAL | SYQGYIDYQF | NWFKPFYFF | KIFMKFAPR | GLKGYKDVY | LGSDVNVPW | AVGYGLKGYK | MEFGGRSGI | FAPRMSLDAL |
| **P. Score** | 0.99 | 0.99 | 0.94 | 0.93 | 0.87 | 0.81 | 0.80 | 0.76 | 0.72 | 0.67 |
| **P. Rank** | 0.01 | 0.01 | 0.01 | 0.01 | 0.04 | 0.06 | 0.12 | 0.11 | 0.14 | 0.14 |
| **Antigenicity** | | | | | | | | | | |
| **Score** | 0.94 | 1.18 | 0.88 | 1.60 | 1.43 | 0.68 | 0.89 | 1.21 | 0.76 | 1.39 |
| **Prospect** | Epitopes are antigenic | | | | | | | | | |
| **Allergenicity** | | | | | | | | | | |
| | Epitopes are non-allergenic | | | | | | | | | |
| **Toxicity** | | | | | | | | | | |
| **Score** | 5.75 E-29 | 2.27 E-30 | 6.73 E-13 | 1.89 E-19 | 5.28 E-14 | 5.28 E-23 | 5.94 E-13 | 5.59 E-27 | 3.67 E-18 | 3.58 E-27 |
| **Prospect** | Epitopes are non-toxic | | | | | | | | | |

HLA: Human leukocyte antigen; P. rank: Percentile rank; P. score: Peptide score

**Table 3.9:** Predicted MHC-II binding epitopes and their antigenicity, toxicity, and allergenicity.

| **Allele** | HLA_DRB 1* 09:01 | HLA_DRB 1* 04:01 | HLA_DQA 1* 05:01 | HLA_DRB 1* 09:01 | HLA_DQA 1* 01:02 |
|---|---|---|---|---|---|
| **Epitopes binding Predictions** | | | | | |
| **Start** | 7 | 74 | 37 | 6 | 11 |
| **End** | 21 | 88 | 51 | 20 | 25 |
| **Length** | 15 | 15 | 15 | 15 | 15 |

| **Epitope Sequence** | ALGLV AATSA PV MAA | DIFNL LSNPS SDKEG | QFNLM AAIDE LPGES | LALGL VAATS APVMA | VAATS APVMA ADYSD |
|---|---|---|---|---|---|
| **P. Rank** | 0.12 | 0.15 | 0.24 | 0.30 | 0.32 |
| | | | **Antigenicity** | | |
| **Score** | 0.4018 | 0.5595 | 0.7497 | 0.5157 | 0.5207 |
| **Prospect** | | | Epitopes are antigenic | | |
| | | | **Allergenicity** | | |
| | | | Epitopes are non-allergenic | | |
| | | | **Toxicity** | | |
| **Score** | 2.59E-14 | 3.88E-12 | 3.70E-19 | 3.59E-13 | 1.40E-05 |
| **Prospect** | | | Epitopes are non-toxic | | |

HLA: Human leukocyte antigen; P. rank: Percentile rank

**Table 3.10:** Predicted B-cell epitopes and their antigenicity, toxicity, and allergenicity.

| **Epitopes binding Predictions** | | | | | |
|---|---|---|---|---|---|
| **Epitope Sequence** | QGYIDYQ FGMDDK NTA | KEKIFMK FAPRMSL DA | YQGYIDY QFGMDD KNT | EGLAGKT TGFGHYL AV | GESSHDY LEMEFGG RS |
| **Start** | 7 | 74 | 37 | 6 | 11 |
| **End** | 21 | 88 | 51 | 20 | 25 |
| **Length** | 15 | 15 | 15 | 15 | 15 |
| **P. Rank** | 0.12 | 0.15 | 0.24 | 0.30 | 0.32 |
| **Antigenicity** | | | | | |
| Epitopes are antigenic | | | | | |
| **Allergenicity** | | | | | |
| Epitopes are non-allergenic | | | | | |
| **Toxicity** | | | | | |
| **Score** | 2.59E-14 | 3.88E-12 | 3.70E-19 | 3.59E-13 | 1.40E-05 |
| **Probability** | | | Epitopes are non-toxic | | |

P. rank: Percentile rank

### 3.3.3. Characteristics evaluation and physiochemical properties of OVC

The different physiochemical properties of the OVC as well as the antigenicity, allergenicity, and toxicity analysis of the modified OVC were checked using different tools. The physicochemical properties of the OVC were examined with the help of the

ExPASy Protparam tool, and SOLpro of the SCRATCH protein predictor tool. The amino acid composition, molecular weight, theoretical isoelectric point (pI), atomic composition, expected half-life *in vitro* and *in vivo*, instability index, extinction coefficient, Grand average of hydropathicity (GRAVY), aliphatic index, the solubility of OVC when overexpressed of the vaccine construct are shown in table 3.11. Vaccines must not cause any allergic reaction in the body and must have a strong antigenic effect. Safe vaccines must not be toxic, and they must be completely foreign particles for the body to generate significant immunogenicity. Therefore, antigenicity, allergenicity, and toxicity of the whole vaccine construct analysis were also done which is shown in Table 3.11 in detail.

### 3.3.4. Two and three-dimensional structures of OVC

The SOPMA server was used to determine the secondary structure of the construct. The accuracy of SOPMA's predictions is higher than 80%. Two-dimensional structural parameters were taken out and analyzed to learn more about the vaccine's composition and how well it works. For OVC, the SOPMA server projected 21.93% α-helix (h), 4.83% β-turn (t), 44.61% random coils (c), and 28.62 % extended strand (e) tabulated (Table 3.11 & Figure 3.2). GalaxyRefine tool was used to create a 3D structure for the vaccine construct. The tool predicted five models for OVC. However, “Model 2” was picked based on its quality factor from ERRAT, the Ramachandran plot analysis from PROCHECK, and the Z-score values from ProSA-Web. Figure 3.3 shows the three-dimensional structure of the “Model 2” of the vaccine construct. The Ramachandran plot for the selected model of OVC showed that 86.3% of the residues were in the most preferred regions, 11.0% were in additional allowed regions, 1.3% were in generously allowed regions, and only 1.3% were in disallowed regions (Figure 3.4 (A)). “Model 2” had the highest ERRAT quality factor of 91.6% (Figure 3.4 (B)). Lastly, the Z-score for the selected model for OVC was found on the ProSA-Web was -5.62 (Figure 3.5). All the values have shown that "Model 2" is of good quality.

**Table 3.11:** OmpK vaccine construct (OVC) physiochemical properties and characteristics.

| **Physiochemical Properties** | | | | |
|---|---|---|---|---|
| **Parameters** | | | **Results** | |
| Total no. of amino acids | | | 269 | |
| Molecular weight | | | 30206.85 | |
| Theoretical isoelectric point (pI) | | | 5.64 | |
| Negative charged residues (Asp+Glu) | | | 31 | |
| Positively charged residues (Arg+Lys) | | | 23 | |
| Formula | | | $C_{1379}H_{2015}N_{347}O_{402}S_{10}$ | |
| Total no. of atoms | | | 4153 | |
| Instability index | | | 12.88 | |
| Aliphatic index | | | 65.99 | |
| Grand average of hydropathicity | | | -0.377 | |
| Solubility | | | Insoluble | |
| **Characteristics** | | | | |
| **Antigenicity** | | **Allergenicity Probability** | **Toxicity** | |
| **Score** | **Probability** | | **Score** | **Probability** |
| 0.6302 | Antigenic | Non-Allergen | $5.36x10^{-7}$ | Non-toxic |

**Table 3.12:** OmpK vaccine construct's (OVC) secondary structural characteristics.

| **Parameters** | **SOMPA Server** | |
|---|---|---|
| | **Amino Acids** | **Percentage (%)** |
| **Alpha helix (h)** | 59 | 21.93 |
| **Beta turn (t)** | 13 | 4.83 |
| **Random coil (c)** | 120 | 44.61 |
| **Extended strand (e)** | 77 | 28.62 |

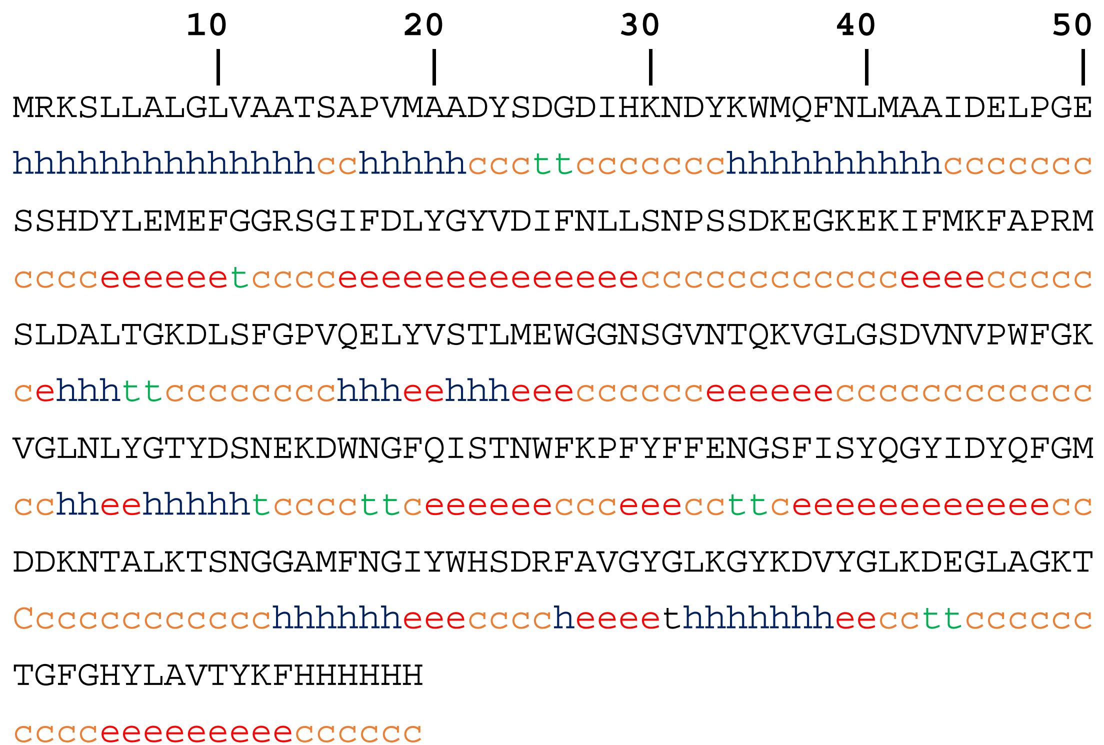



**Figure 3.2: OVC protein sequence and two-dimensional structure.** The two-dimensional structure's alpha helix (h), beta-turn (t), random coil (c), and extended strand (e) are shown in blue, green, orange, and red respectively.

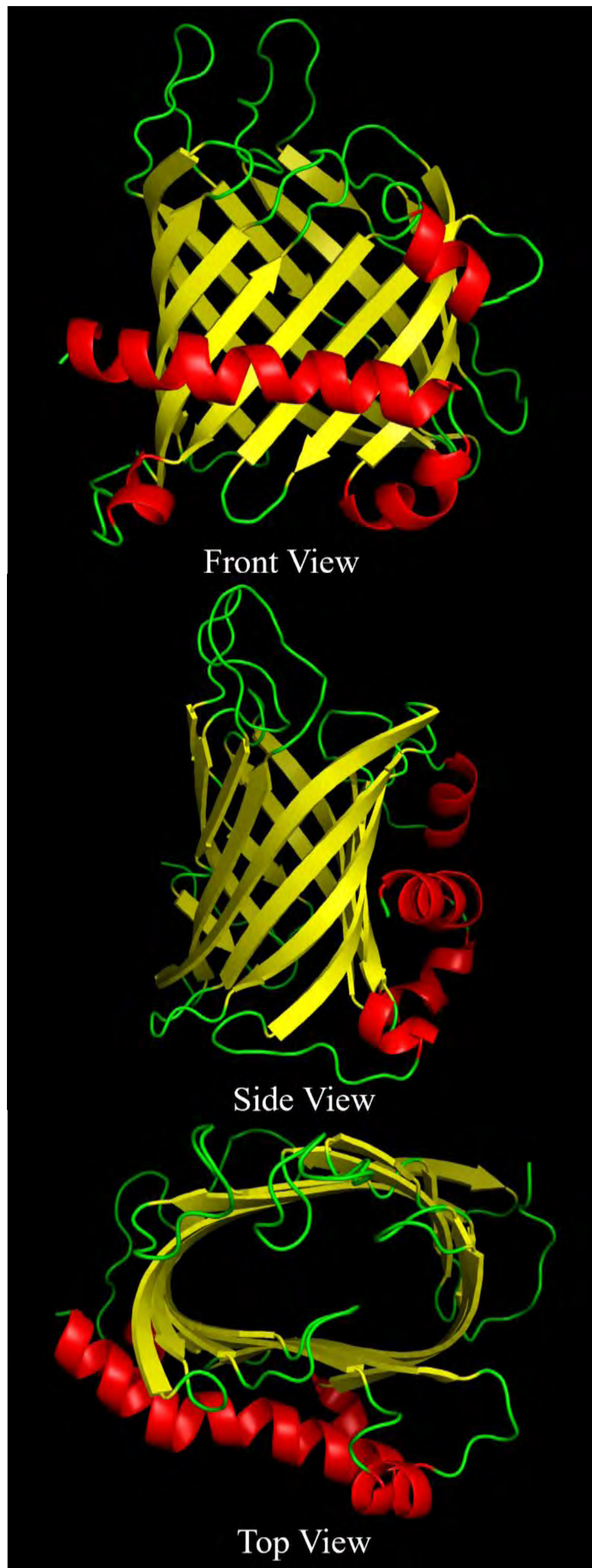
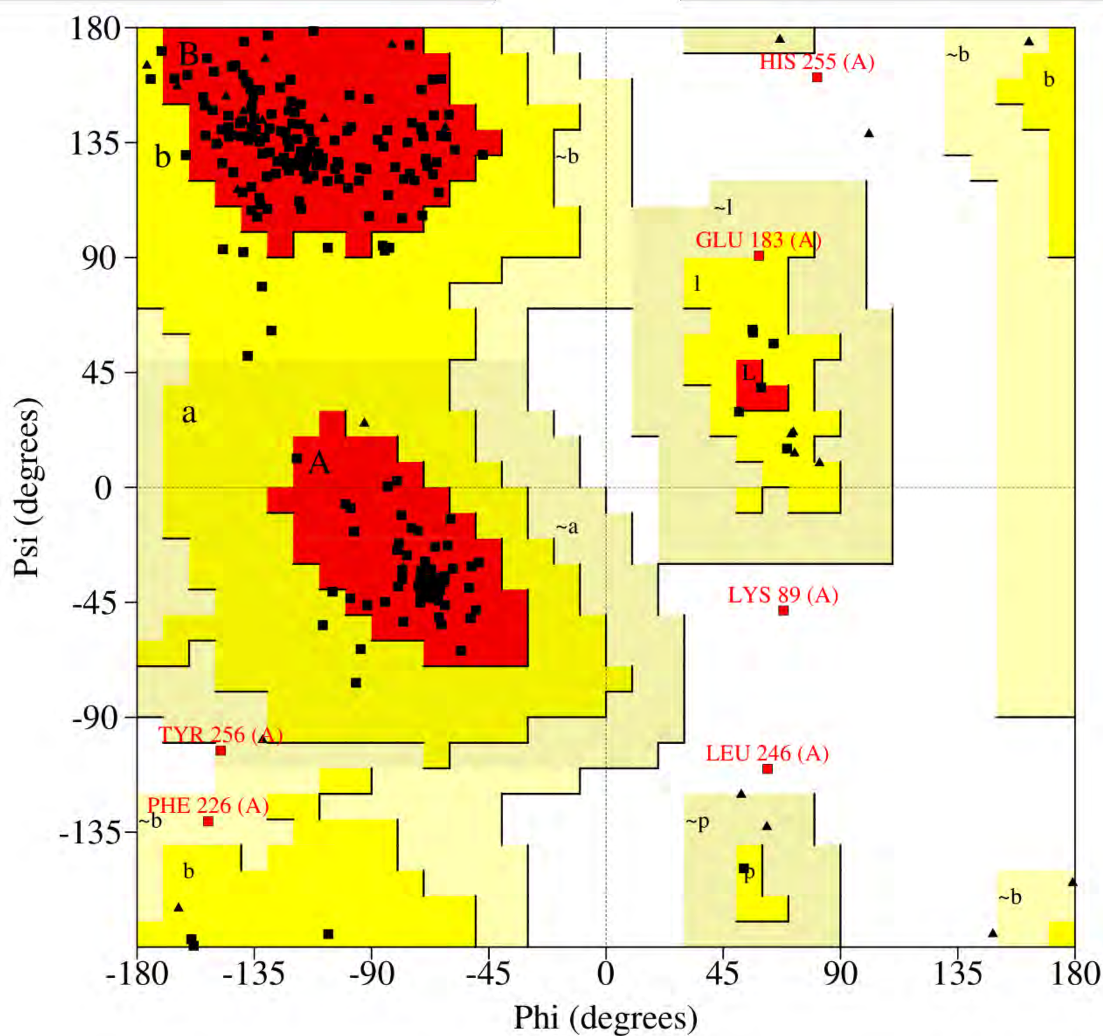
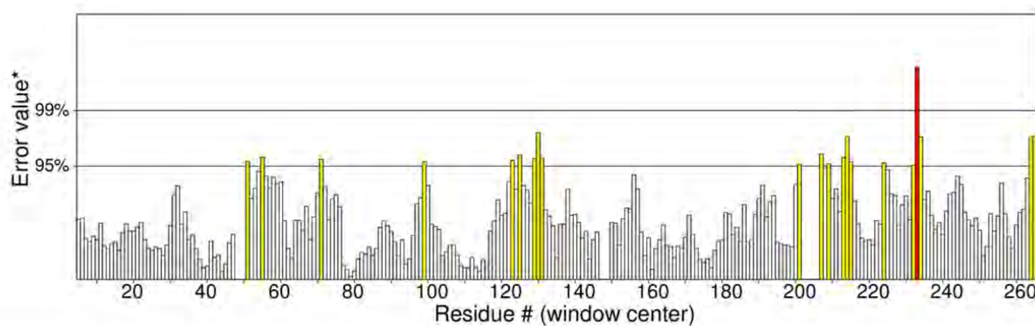



**Figure 3.3: Three-dimensional structure of OVC**




**(A)**




* On the error axis two lines are drawn to indicate the confidence with which it is possible to reject regions that exceed the error value.

** Expressed as the percentage of the protein for which the calculated error value falls between the 95% rejection limit. Good high-resolution structures generally produce values around 95% or higher. For lower resolutions (2.5 to 3 ) the average overall quality factor is around 91%.

**(B)**

**Figure 3.4: OVC quality validation. (A).** Ramachandran *p*lot for the "Model 2" of the OVC. **(B)** ERRAT error prediction (91.6%).

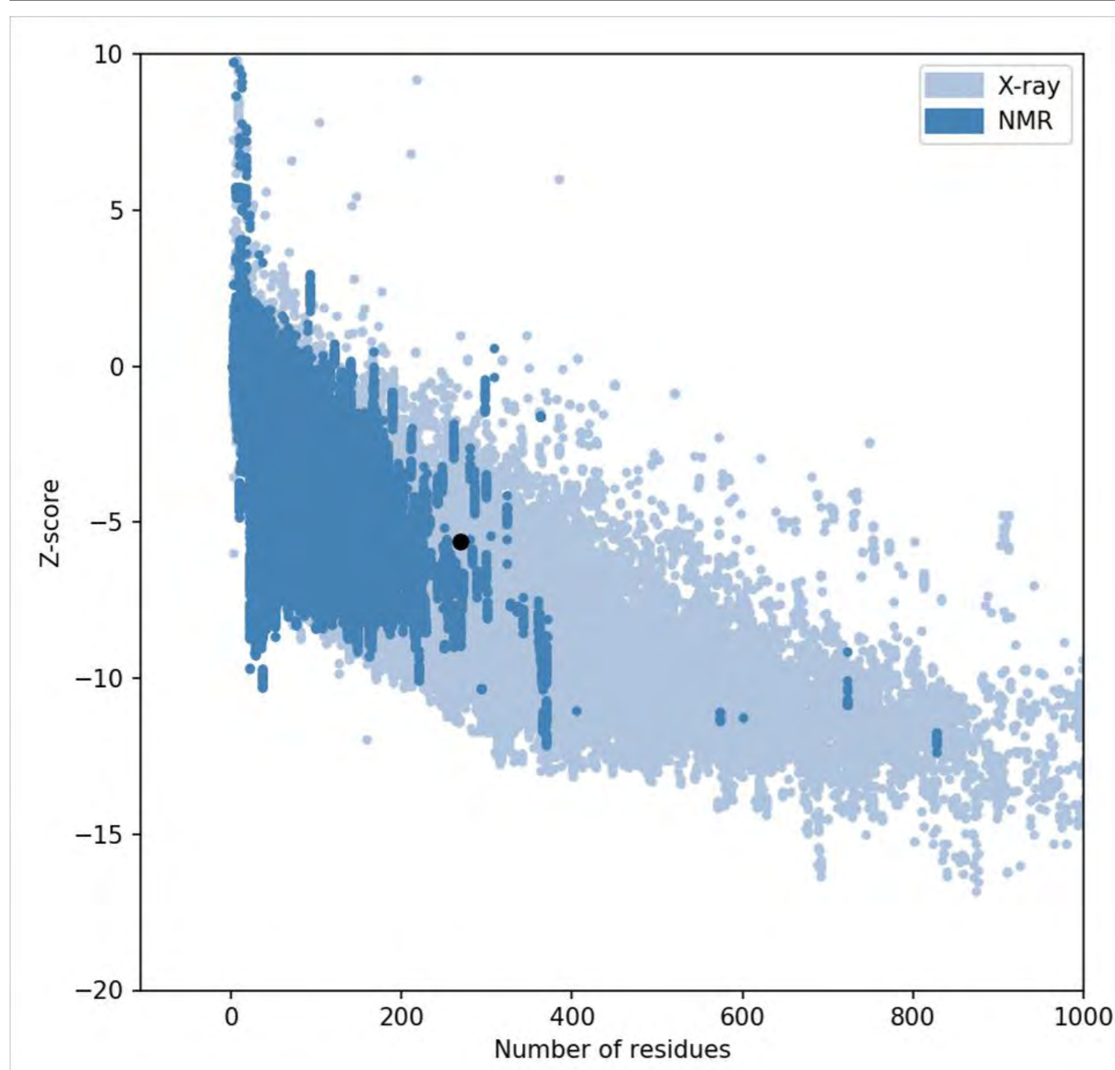
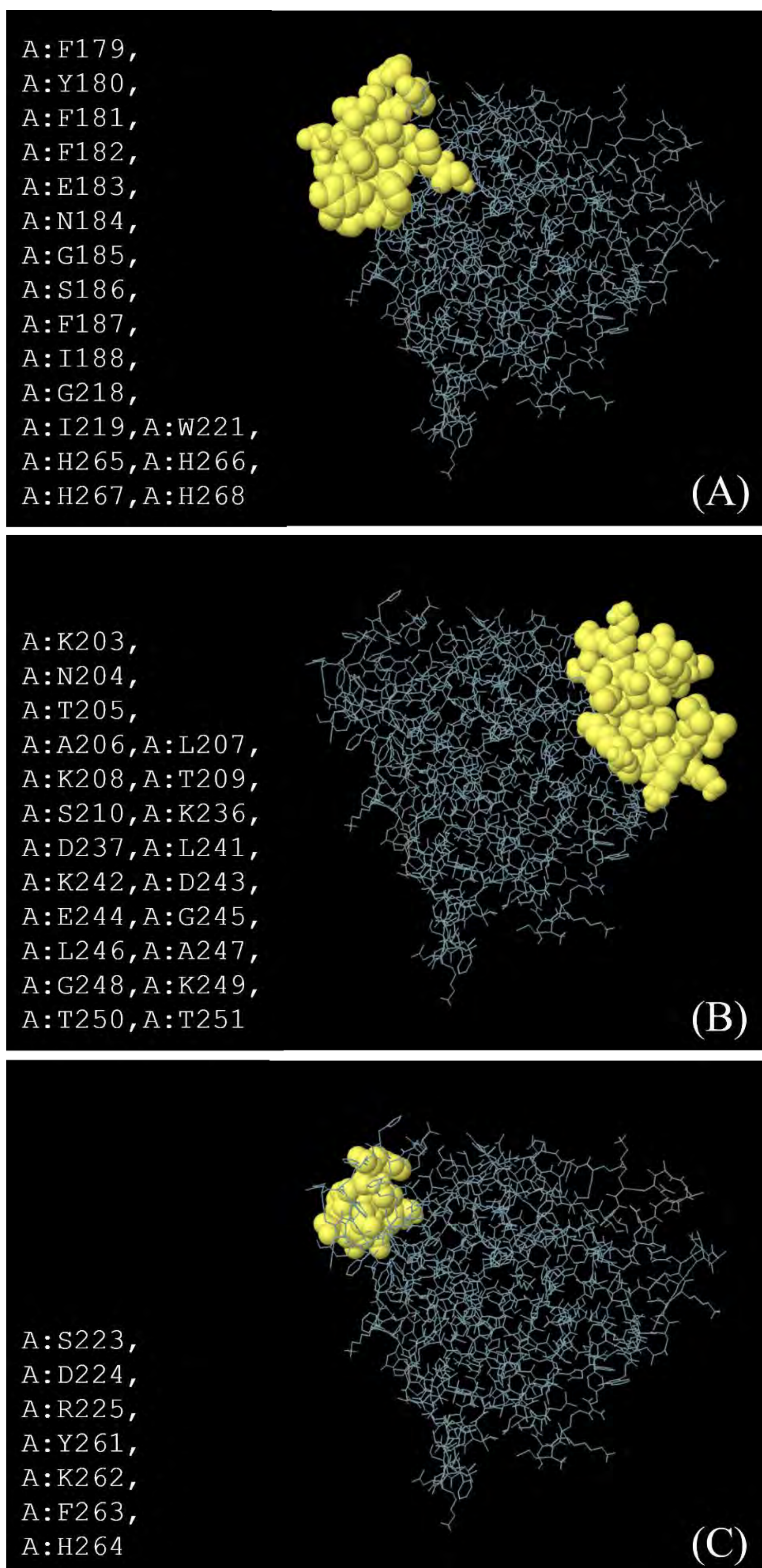
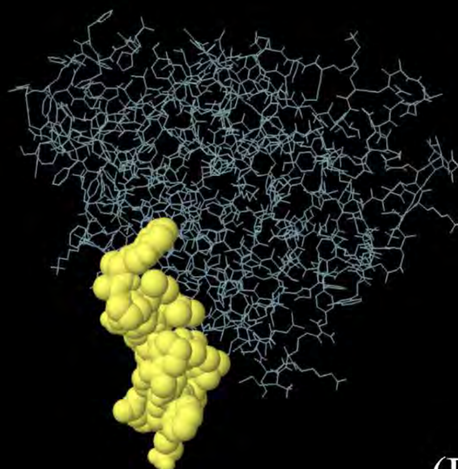


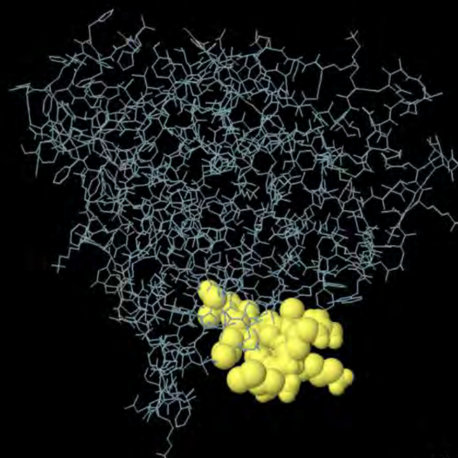

**Figure 3.5: ProSA web analysis for quality validation for OVC "Model 2".** The Z-score was -5.62.

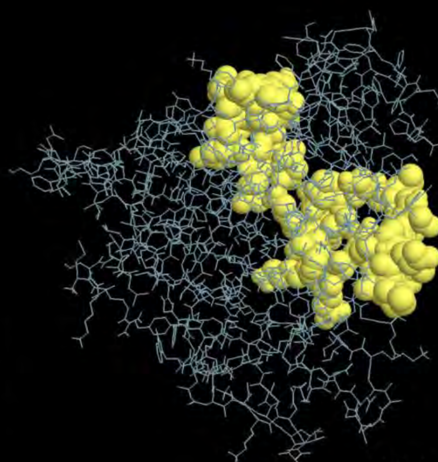
### 3.3.5. Detection of discontinuous B-cell epitopes

The production of antibodies that are specific to a pathogen is dependent on the activation of B-cells, which is an essential step in the development of an immune response. The discontinuous epitopes for the OVC were found by utilizing the default settings for the ElliPro webserver. As a result of this, the software identified nine discontinuous epitopes for the OVC (Figure 3.6 (A-I)), which are enough epitopes that are needed to bind to a designed vaccine to get the immune system to work properly.


A:F179,
A:Y180,
A:F181,
A:F182,
A:E183,
A:N184,
A:G185,
A:S186,
A:F187,
A:I188,
A:G218,
A:I219,A:W221,
A:H265,A:H266,
A:H267,A:H268
(A)
A:K203,
A:N204,
A:T205,
A:A206,A:L207,
A:K208,A:T209,
A:S210,A:K236,
A:D237,A:L241,
A:K242,A:D243,
A:E244,A:G245,
A:L246,A:A247,
A:G248,A:K249,
A:T250,A:T251
(B)
A:S223,
A:D224,
A:R225,
A:Y261,
A:K262,
A:F263,
A:H264
(C)


A:S64,
A:G65,
A:I66,
A:L102,A:D103,
A:A104,A:L105,
A:T106,A:G107,
A:K108,A:D109,
A:L110,A:S111,
A:F112,A:G113,
A:P114,A:V115,
A:Q116,A:E117,
A:L118,A:V143,
A:N144,A:V145,
A:P146,A:W147
(D)


A:M1,
A:R2,
A:K3,
A:S4,
A:L6,
A:A7,
A:L8,
A:V11,
A:A12
(E)


A:D22,A:Y23,
A:D25,A:G26,
A:D27,A:I28,
A:H29,A:K30,
A:N31,A:D32,
A:Y33,A:D45,
A:E46,A:L47,
A:S51,A:S52,
A:H53,A:D54,A:N77,
A:L78,A:L79,A:S80,
A:N81,A:P82,A:S83,
A:S84,A:D85,A:K86,
A:E87,A:G88,A:K89,
A:E90,A:K91,A:I92
(F)

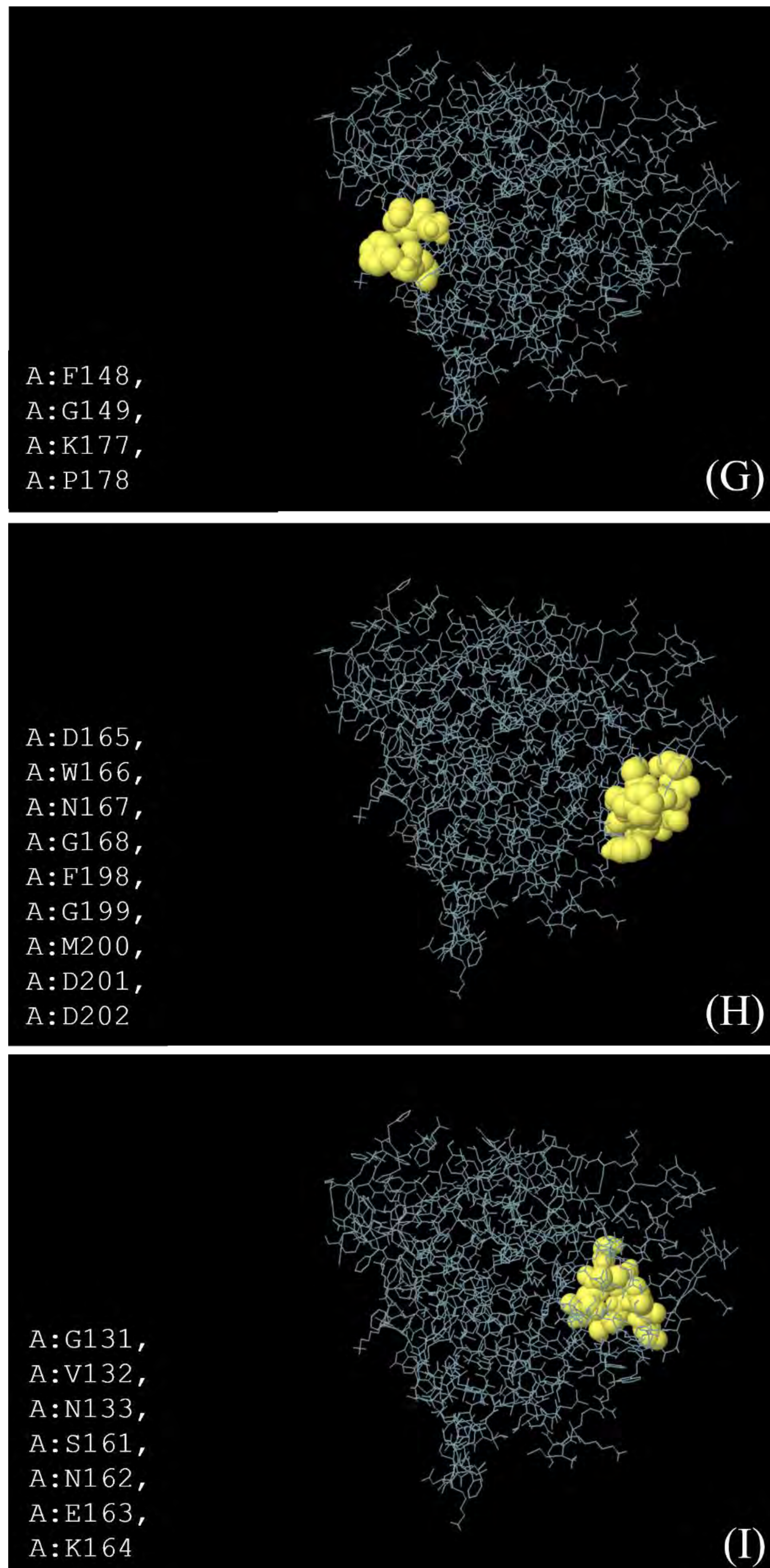


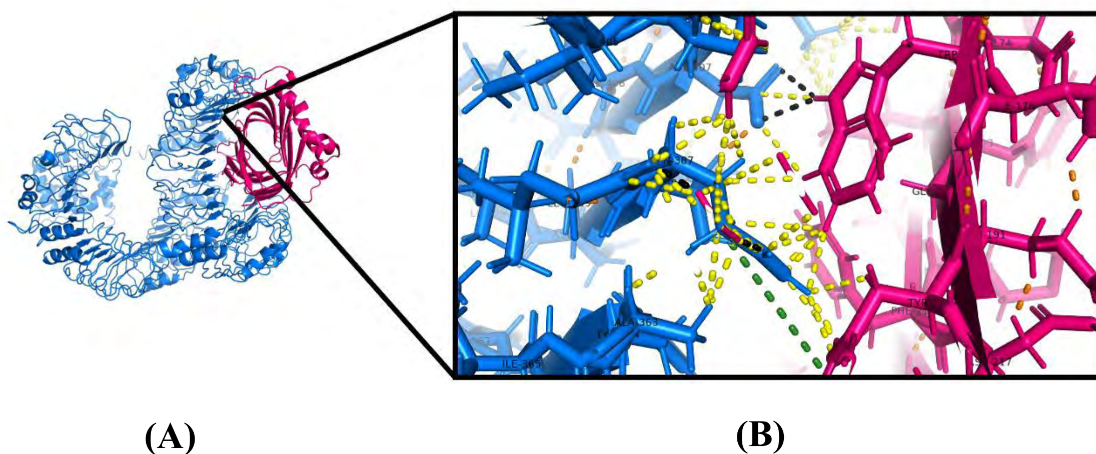

**Figure 3.6: Nine discontinuous B-cell epitopes on OVC Model 2. (A-I)** Shows epitopes name (white), discontinuous B-cell epitopes (yellow), and OVC (grey sticks).

### 3.3.6. Molecular docking of OVC

ClusPro 2.0 Protein-Protein Docking server was used for molecular docking analysis of the vaccine candidate against TLR-5 (RCSB PDB ID: 3J0A). Twenty-nine docked structures because of possible interactions between TLR-5 and the OVC were generated as a result. Further analysis was done with the PatchDock web server and its refinement and scoring with FireDock. The best-docked model (Figure 3.7) with the lowest energy -1405 was chosen. The values of the binding affinity, intermolecular energy, attractive Van der Waal's forces (Attractive VdW), energy contributed by hydrogen bonds (HB), and highest global energy by the docked model were also considered during the selection of the best-docked model. The highest global energy, Van der Waal's forces (Attractive VdW), and energy contributed by hydrogen bonds (HB) are shown in Table 3.13.

**Table 3.13:** Values on the basis of which the best docking complex is chosen.

| Ligand | Receptor | Global Energy | Attractive VdW | HB |
|---|---|---|---|---|
| OVC | TLR-5 | -57.96 | -43.82 | -2.89 |



**(A)** **(B)**

**Figure 3.7: Molecular docking of "Model 2" of OVC with TLR-5. (A)** Docked complex of OVC (hot pink) and TLR-5 (marine blue). **(B)** Interacting amino acids (black), polar contact (black dashes), any contacts within 3.0 Å (yellow dashes), all pi interactions (forest green dashes), and Van der Waal's forces with distance ratio < 0.75 (orange dashes).

### 3.3.7. Codon optimization and cloning of OVC

GenSmart$^{TM}$ Codon Optimization web server was used to perform codon optimization on the OVC construct per the plant codons. This was done to facilitate expression

analysis. The GC content found in the OVC was 41.02%. This shows that potential OVC may show a significant expression in the host system (*Nicotiana tabacum*). Figure 3.8 shows the alignment of DNA sequence retrieved from NCBI and the DNA sequence after codon optimization and Figure 3.9 shows the alignment of both NCBI retrieved and codon optimized translated protein sequence.

```
OS ATGCGTAAATCACTTTTAGCTCTAGGCCTAGTGGCTGCAACTTCTGCTCCT 51
CO ATGAGAAAGTCTTTGTTGGCTCTCGGATTGGTGGCAGCTACTTCAGCTCCT 51
   ***** ** **  * ** ***** **  * ***** ** ***** ******

OS GTTATGGCCGCTGACTATTCAGATGGCGACATCCATAAAAACGATTACAAA 102
CO GTTATGGCTGCTGACTATTCAGATGGTGATATTCATAAGAATGATTACAAG 102
   ******** ***************** ** ** ***** ** ********

OS TGGATGCAATTTAACCTAATGGCTGCAATCGATGAATTACCAGGTGAATCA 153
CO TGGATGCAATTTAACCTTATGGCTGCAATCGACGAACTGCCAGGAGAGAGC 153
   ***************** ************** *** * ***** **

OS TCGCATGATTACCTAGAGATGGAATTTGGCGGCCGCTCAGGGATTTTTGAT 204
CO TCACATGATTACTTGGAGATGGAGTTTGGAGGACGATCCGGGATTTTTGAT 204
   ** ********* * ******** ***** ** ** ** ************

OS CTGTACGGTTACGTTGATATCTTTAACCTGCTAAGCAACCCAAGCAGTGAC 255
CO CTCTATGGTTATGTGGATATCTTCAATTTGCTTAGTAATCCATCCTCTGAT 255
   ** ** ***** ** ******** **  **** ** ** ***  *  ***

OS AAAGAAGGTAAAGAAAAAATCTTTATGAAATTTGCACCTCGTATGTCACTA 306
CO AAGGAGGGTAAGGAAAAGATTTTCATGAAGTTTGCACCAAGAATGTCTTTG 306
   ** ** ***** ***** ** ** ***** ********  * *****  *

OS GATGCACTTACAGGTAAAGATTTGTCTTTCGGGCCAGTGCAAGAGTTGTAT 357
CO GATGCTCTTACCGGAAAGGATCTTAGTTTTGGTCCCGTTCAAGAGCTTTAT 357
   ***** ***** ** ** *** *   *** ** ** ** ****** * ***

OS GTCTCTACGCTAATGGAATGGGGTGGTAACTCTGGCGTTAACACTCAAAAA 408
CO GTTAGTACTCTTATGGAGTGGGGTGGAAATTCAGGGGTTAACACTCAAAAG 408
   **   *** ** ***** ******** ** ** ** **************

OS GTGGCCTTGGCTCTGATGTGAATGTGCCTTGGTTTGGTAAAGTGGGTTTA 459
CO GTTGGATTGGGTTCCGATGTTAACGTTCCTTGGTTCGGTAAGGTTGGTTTG 459
   ** **  * ** ** ***** ** ** ******** ***** ** *****

OS AACCTTTACGGTACTTATGATTCAAATGAGAAAGATTGGAACGGCTTCCAA 510
CO AATTTGTATGGAACTTATGATTCCAACGAAAAAGATTGGAATGGATTCCAG 510
   **  * ** ** *********** ** ** *********** ** *****

OS ATTTCAACCAACTGGTTCAAACCTTTTTACTTCTTTGAAAACGGCTCGTTT 561
CO ATTTCTACTAACTGGTTTAAACCTTTTTATTTTTTTGAGAATGGAAGCTTC 561
   ***** ** ******** *********** ** ***** ** **    **

OS ATCTCTTATCAAGGTTACATCGATTACCAATTTGGCATGGATGACAAAAAT 612
CO ATTTCTTACCAGGGGTATATCGATTACCAATTCGGTATGGATGATAAGAAC 612
   ** ***** ** ** ** ************** ** ******** ** **

OS ACTGCACTAAAAACCTCAAATGGTGGTGCAATGTTTAACGGTATTTACTGG 663
CO ACCGCATTGAAGACTAGTAATGGTGGTGCTATGTTCAATGGTATTTACTGG 663
   ** *** * ** **    *********** ***** ** ************

OS CACTCCGATCGCTTTGCTGTTGGCTACGGCCTTAAAGGCTACAAAGATGTT 714
CO CACAGCGATAGGTTTGCTGTTGGTTATGGTTTGAAGGGTTATAAGGATGTG 714
   ***  **** * *********** ** **  * ** ** ** ** *****

OS TATGGTCTGAAAGATGAAGGTCTTGCAGGCAAAACAACTGGCTTTGGTCAC 765
CO TATGGTTTGAAGGATGAGGGACTTGCAGGTAAAACAACAGGATTTGGACAT 765
   ****** **** ***** ** ******** ******** ** ***** **

OS TACCTAGCAGTAACTTACAAGTTCCATCACCATCACCATCAC         807
CO TATCTTGCTGTTACTTATAAATTTCATCATCACCATCATCAT         807
   ** ***** ** ***** ** ** ***** ** ** *****
```

**Figure 3.8: CLUSTAL O (1.2.4) multiple sequence alignment of DNA sequence after codon optimization.** OS; Original sequence, CO; Codon optimized sequence.

**CLUSTAL O (1.2.4) multiple sequence alignment**

```
OS MRKSLLALGLVAATSAPVMAADYSDGDIHKNDYKWMQFNLMAAIDELPGE 50
CO MRKSLLALGLVAATSAPVMAADYSDGDIHKNDYKWMQFNLMAAIDELPGE 50
   **************************************************
OS SSHDYLEMEFGGRSGIFDLYGYVDIFNLLSNPSSDKEGKEKIFMKFAPRM 100
CO SSHDYLEMEFGGRSGIFDLYGYVDIFNLLSNPSSDKEGKEKIFMKFAPRM 100
   **************************************************
OS SLDALTGKDLSFGPVQELYVSTLMEWGGNSGVNTQKVGLGSDVNVPWFGK 150
CO SLDALTGKDLSFGPVQELYVSTLMEWGGNSGVNTQKVGLGSDVNVPWFGK 150
   **************************************************
OS VGLNLYGTYDSNEKDWNGFQISTNWFKPFYFFENGSFISYQGYIDYQFGM 200
CO VGLNLYGTYDSNEKDWNGFQISTNWFKPFYFFENGSFISYQGYIDYQFGM 200
   **************************************************
OS DDKNTALKTSNGGAMFNGIYWHSDRFAVGYGLKGYKDVYGLKDEGLAGKT 250
CO DDKNTALKTSNGGAMFNGIYWHSDRFAVGYGLKGYKDVYGLKDEGLAGKT 250
   **************************************************
OS TGFGHYLAVTYKFHHHHHH                                269
CO TGFGHYLAVTYKFHHHHHH                                269
   *******************
```

**Figure 3.9: CLUSTAL O (1.2.4) multiple sequence alignment of translated protein sequence after codon optimization.** OS; Original sequence, CO; Codon optimized sequence.

# *CLONING AND TRANSFORMATION OF OMPK*

*Chapter 4*

# 4.1. Introduction

## 4.1.1 Molecular cloning

In modern molecular biology, molecular cloning emerged as a pivotal experimental technique which has proven itself critically instrumental in driving biotechnological advances. The discovery of restriction endonucleases (bacterial enzymes) embarked on the development of techniques in molecular cloning. These bacterial enzymes based on nucleotide sequences cleave DNA at predetermined specific sites allowing researchers to fragment large DNA into manageable pieces and ligate into vectors (plasmids) with the help of DNA ligase enzymes. Plasmids are physically distant circular chromosomal DNA with independent replication capabilities (Bertero *et al*., 2017; Green & Sambrook, 2020a; Maheshwari *et al*., 2022; P. Yan *et al*., 2020).

Molecular cloning is a series of experimental techniques employed to generate a population of organisms harboring identical recombinant DNA molecules. In molecular cloning, the recombinant DNA (rDNA) is first synthesized *in vitro* and then transferred for its replication into a host organism, which along with its proliferation orchestrates the recombinant DNA replication. Molecular cloning is typically achieved using easily cultivable and non-pathogenic bacterial strains such as *Escherichia coli* in laboratories. A single genetically modified *E. coli* cell harboring the desired rDNA molecule can proliferate exponentially to yield unlimited copies of identical DNA. Due to this property molecular cloning can also be analogized to an "*in vivo* polymerase chain reaction (PCR)" but it has better fidelity, higher yield, lower cost, and more flexibility as compared to PCR (Bertero *et al*., 2017; H. Jiang *et al*., 2022; Maheshwari *et al*., 2022; Parui *et al*., 2022; P. Yan *et al*., 2020).

## 4.1.2. Methods of molecular cloning

Different molecular cloning techniques have been developed, each with distinct advantages and disadvantages. Some of them such as PCR cloning, ligation-independent cloning, seamless cloning, golden gate cloning, and recombinant cloning are explained one by one (Bertero *et al*., 2017; P. Yan *et al*., 2020).

### 4.1.2.1. PCR cloning

In PCR cloning a PCR-generated DNA fragment is directly ligated into a vector without restriction enzymes involvement. This method is also known as "TA" cloning as

adenine (A) residue is added at the 3' end of the desired DNA fragment with the help of *Taq* polymerase PCR during amplification. This A-tailed DNA fragment is then easily ligated into "T-tailed" vectors. This type of cloning is time-consuming and laborious. Another limiting factor is the availability of relatively few restriction sites for restriction enzymes especially when the scientists need to insert multiple fragments in a single complex plasmid (Bertero *et al*., 2017; Green & Sambrook, 2021; Lampropoulos *et al*., 2013; J. Wang *et al*., 2014; P. Yan *et al*., 2020; M.-Y. Zhou & Gomez-Sanchez, 2023).

#### 4.1.2.2. Ligation independent cloning (LIC)

In this type of cloning short DNA sequences are added to a cloning fragment with the help of modified primers during amplification by PCR. The cloning fragment is homologous to the destination vector. Then 3' and 5' endonucleases are used to create complementary cohesive ends in both insert and vector. The cohesive end containing the insert and vector are then mixed to get an annealed product, but this product has four single-stranded nicks which can be easily repaired by host DNA repair machinery. This method results in a "scar-free" vector which does not have any unwanted sequences or new restriction sites. This method is also time-consuming and laborious (Bertero *et al*., 2017; Cohen *et al*., 1973; Jeong *et al*., 2012; Lampropoulos *et al*., 2013; M. Z. Li & Elledge, 2007; J. Wang *et al*., 2014; P. Yan *et al*., 2020).

#### 4.1.2.3. Seamless cloning

It is a set of techniques that involve the insertion of one or more DNA fragments into a vector in a single isothermal reaction in a manner devoid of sequence-dependent limitations or undesired scars such as the Gibson Assembly Method, Gibson-driven Hot fusion, In-fusion, and TEDA. In these methods, up to ten DNA fragments can easily be inserted into a vector based on 15-20 bp homologous ends. The endonuclease activity, ligation, and repair are analogous to the LIC method. Because of sequence independence, there are currently no standard protocols available for overlapping sequence designing. This method is not free of restriction enzymes as the linearization of the destination vector is required which can be achieved by PCR or digestive enzymes (Benoit *et al*., 2016; Bertero *et al*., 2017; Fu *et al*., 2014; Gibson *et al*., 2009; Lampropoulos *et al*., 2013; Motohashi, 2017; Sleight *et al*., 2010; J. Wang *et al*., 2014; Xia *et al*., 2019; P. Yan *et al*., 2020; Y. Zhang *et al*., 2014; B. Zhu *et al*., 2007).

#### 4.1.2.4. Golden Gate cloning

This type of cloning also known as Golden Gate assembly/shuffling and capable of accurately and efficiently assembling multiple DNA fragments by using “Type II” restriction enzymes. The limiting factor of this cloning technique is that the GoI must be free of restriction sites specific to enzymes used for cutting in this method. However, the recognition sequence for these enzymes is less than seven base pairs resulting in the frequent presence of these restriction sites within the DNA sequence to be cloned. So the cloning of longer DNA fragments and multiple fragments by this method is limited due to this problem (Bertero *et al.*, 2017; Engler *et al.*, 2008, 2009; Engler & Marillonnet, 2011; P. Yan *et al.*, 2020).

#### 4.1.2.5. Recombinational cloning

This type of cloning uses enzymes which can swap DNA pieces between the two DNA molecules containing the appropriate sequences. These enzymes are named site-specific DNA recombinases and specific sites (*att* sites) based on which they swap the DNA pieces are named recombination sites. The most well-known cloning method of this type is the “Gateway Cloning System” developed by “Invitrogen/Life Technologies” (Bertero *et al.*, 2017; Hartley *et al.*, 2000; G. Qin *et al.*, 2022).

The Gateway Cloning System is a cloning technique which uses two main and important enzyme mixes namely “BP Clonase” and “LR Clonase. These enzyme mixes swap a DNA fragment across the twenty-five bp-specific recombination sites. First, with PCR on either side of GoI, the appropriate recombination sites were inserted. Then the resulting PCR product having the recombination sites is recombined with the Donor vector by the action of BP clonase enzymes to get an Entry clone (in the market enormous collection is available). After that Entry clone is combined with the Destination vector by the action of LR clonases to make the final construct (Expression vector). Even though Gateway cloning has been extensively rummaged in many experiments but has four key drawbacks: (i) the two-step BP and LR recombination reactions-based cloning is laborious and extensive process; (ii) the undesirable 25 bp junk recombination site sequence (scar) is left in final expression vector; (iii) the multiple fragments assembly is comparatively not possible; and (iv) the enzyme mixes are expensive, particularly for developing countries’ laboratories (Bertero *et al.*, 2017; Walhout *et al.*, 2000; P. Yan *et al.*, 2020).

All the established molecular cloning experiments can be divided into the following stages: a) the host organism selection, b) cloning vector selection, c) vector preparation, d) gene of interest "GoI" preparation, e) rDNA generation, f) rDNA introduction into the host, g) transformed clones selection and screening, and h) rDNA/desired expansion and isolation (Bertero *et al*., 2017; P. Yan *et al*., 2020).

#### 4.1.3. Transformation of rDNA into host organisms

Different methods are available for the introduction of rDNA into host organisms depending on the type of host.

##### 4.1.3.1. Introduction of rDNA into bacteria

There are different methods of gene transfer in bacteria such as biolistics methods, electroporation, heat shock method, laser irradiation, mineral nanofibers, shock wave, and sonoporation (Lin *et al*., 2010; Mahdavi *et al*., 2022; Prakash *et al*., 2011; Shark *et al*., 1991; Song *et al*., 2007; Tiflova *et al*., 1997; Wilharm *et al*., 2010). The widely used methods for the introduction of rDNA into bacteria are the heat shock method and electroporation (Dower *et al*., 1988; Fiedler & Wirth, 1988; Green & Sambrook, 2020b; Jin *et al*., 2020; Prakash *et al*., 2011; Tang *et al*., 1994; Ueda *et al*., 1991).

- **Heat shock method:** This method is the most used method due to its simplicity for the rDNA introduction into bacteria. In this method cells under frigid conditions pretreated with chemicals (often calcium chloride) followed by a heat shock pulse. This treatment increases the cell membrane permeability of bacteria for the rDNA. Plasmids of size up to 10kb can be inserted into bacteria efficiently by this method (Bertero *et al*., 2017; Jin *et al*., 2020; Prakash *et al*., 2011; Tang *et al*., 1994).
- **Electroporation:** Electroporation has higher efficiency as compared to the heat shock method. In this method, the bacterial cells are subjected to transient electric shock that creates minute pores in the cell membrane of bacteria which facilitates the entry of rDNA into the bacterial cell. Large plasmids can easily be introduced into bacteria by this method (Bertero *et al*., 2017; Dower *et al*., 1988; Fiedler & Wirth, 1988; Green & Sambrook, 2020b; Prakash *et al*., 2011; Ueda *et al*., 1991).

##### 4.1.3.2. Introduction of rDNA into plants

Genetic transformation methods for plants are usually divided into direct and indirect methods. Indirect transformation methods are those which use bacteria or viruses, also

known as biological methods. While physical methods are regarded as direct methods which involve cell wall penetration (Keshavareddy *et al*., 2018; A. Q. Rao *et al*., 2009; Rivera *et al*., 2012). Different direct and indirect transformation methods are *Agrobacterium*-mediated, biolistics, electrophoresis, electroporation, laser microbeams, lipofection, microinjection, microinjection, sonication, silicon carbide whisker-mediated, ultrasound/shockwave, vacuum infiltration, and virus-based (Keshavareddy *et al*., 2018; Rakoczy-Trojanowska, 2002; Rivera *et al*., 2012). Among all the above methods the most popular genetic transformation methods for plants are *Agrobacterium*-mediated, biolistics, and electroporation.

- ***Agrobacterium*-mediated:** In this method a pathogenic bacterium is used for the introduction of GoI carrying plasmid. Soil bacteria's (*Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*) innate ability to transform plants has been manipulated for the development of transgenic plants. In the realm of agriculture and biotechnology, the genetic transformation of plants has been viewed as a significant prospect for several reasons such as economic benefits, environmental benefits, improved crop traits, nutritional enhancement, reduced pesticide use and research advancements. In the 1970s the potential of utilizing *A. tumefaciens* for the rational integration of rDNA/exogenic DNA revolutionized the field of plant transformation. *Agrobacterium* was the rational and preferred transformation choice as it naturally and stably integrates T-DNA into the plant cells at nuclear level derived from its tumor-inducing (Ti) plasmid (Chilton *et al*., 1977; Keshavareddy *et al*., 2018; Naik, 2022; Spiegel *et al*., 2015). So far, this method is the most effective and commonly used technique for generating many transgenic plant species including dicots and monocots because a) the integration of genome is precise, simple, and stable, b) the copy number is low, c) integration is inherited into next generation, d) high efficiency, and d) various cell types can be used for transformation. Reliable and efficient protocols have been established for several dicotyledonous and monocotyledonous species for *Agrobacterium*-mediated transformation. Species such as banana, corn, hybrid larch, rice, ryegrass, sugarcane, tall fescue, and wheat are some examples which are transformed with *A. tumefaciens* (Affandi *et al*., 2020; Bettany *et al*., 2003; Dang *et al*., 2022; Grogg *et al*., 2022; Hayta *et al*., 2021; M. Kang *et al*., 2022; Keshavareddy *et al*., 2018; Masters *et al*., 2020; Pratiwi & Surya, 2020; Sabu *et al*., 2021; Takamizo & Sato, 2020; Villao *et al*., 2021). However, significant challenges persist for genotype-independent transformation of

several economically important crop and forest species (De La Riva *et al*., 1998; Gelvin, 2003b, 2003a, 2010; Imani & Kogel, 2020; Keshavareddy *et al*., 2018; F. Liu *et al*., 2020; Naik, 2022).

- **Biolistics:** Particle bombardment also known as biolistics particle bombardment or biolistics or gene gun technique was initially introduced in 1987 for the development of transgenic plants (Sanford *et al*., 1987). In this technique accelerated high-density DNA coated carrier particles penetrate the cell and deposit the DNA inside it. For genetic transformation of bacteria, fungi, plants, subcellular organelles, and even animal cells biolistics stands out as the most accepted direct technique. Cells, embryos or callus, meristems, and protoplasts can be potential targets (JA, 1993; Lacroix & Citovsky, 2020; Rakoczy-Trojanowska, 2002; Ramkumar *et al*., 2020; Rivera *et al*., 2012; Sanford, 2000; W. Su *et al*., 2023). This method can also be employed for both chloroplast and nuclear transformation in plants. It is easy, less costly, requires a short processing time, and cell wall pre-treatments are not required. Biolistics can be used to introduce chimeric DNA or multiple genes in a plant without depending on specific sequence vectors of a specific sequence and physiological properties of cells like cell membrane cellular components and electrical potential. However, there are some disadvantages such as DNA can be damaged, low transformation efficiency, and expensive, each biological target requires a different set of optimized parameters for transformation, and requires consumables continuous supply (Boynton *et al*., 1988; JA, 1993; Rivera *et al*., 2012; Rustgi & Luo, 2020; W. Su *et al*., 2023).
- **Electroporation:** Electroporation is a widespread method of transformation at the genetic level. It is a cheap, quick, and simple method that can be applied to plant tissues or protoplasts (Daniell *et al*., 1998; Darmawan *et al*., 2020; Fromm *et al*., 1986; Rivera *et al*., 2012; W. Su *et al*., 2023). It is frequently employed for the delivery of biochemical molecules like DNA, lipids, proteins, and RNA into the interior of the cell. The technique promotes the pore formation on the surface of the cell membrane due to a polarity alteration caused by a local driving electrical force (Bates *et al*., 1983; Darmawan *et al*., 2020; Fromm *et al*., 1986; Hofmann & Evans, 1986; Saulis *et al*., 1991). This method has low efficiency, a laborious protocol for both before and after genetic transformation as it can only be applied to protoplasts, and depends on plants' electrophysiological characteristics (Ozyigit, 2020; Rakoczy-Trojanowska, 2002; A. Q. Rao *et al*., 2009; Rivera *et al*., 2012; Sawahel & Cove, 1992; W. Su *et al*., 2023).

### 4.1.4. Plants as "biofactory" for protein expression

The first attempt to develop a genetic crop was made on maize almost half a century ago, but this attempt was a failure (Coe Jr & Sarkar, 1966). So far different genetically stable transformed plants like canola, cassava, celery, chrysanthemum, grapes, maize, millets, petunia, rice, tobacco, tomato, and wheat were developed (Bhatt *et al*., 2021; Dai *et al*., 2020; Danial *et al*., 2021; Elegba *et al*., 2021; Feng *et al*., 2021; Firsov *et al*., 2020; Hayta *et al*., 2021; HNATIUK *et al*., 2020; Imani & Kogel, 2020; M. Kang *et al*., 2022; Kassahun *et al*., 2021; F. Liu *et al*., 2020; J.-X. Liu *et al*., 2022; Madadi *et al*., 2022; Nakajima *et al*., 2020; Niedbała *et al*., 2021; Ovcharenko *et al*., 2023; Pei *et al*., 2023; Peterson *et al*., 2021; Rivera *et al*., 2012; Sabu *et al*., 2021; Segatto *et al*., 2022; Sood *et al*., 2020; Sustiprijatno *et al*., 2022; Utsumi *et al*., 2022; S. Xu *et al*., 2022). Tomato was the first genetically modified species that was approved by the Food and Drug Administration (FDA) in 1994 for distribution in USA market. The transgenic crops worldwide increased by 113 folds from 1996-2018 including canola, corn, cotton, maize, and soy etc. (Alvarez, 2011; Darbani *et al*., 2008; Fernandez-Cornejo, 2009; Herrera-Estrella *et al*., 2004; ISAAA, 2018; K. Kumar *et al*., 2020; Schlegel, 2007; Van den Eede *et al*., 2004). Furthermore, advances in biotechnology to produce novel recombinant proteins from plants opened the doors for pharmaceutical industry's future in plant biofactories. They are cost effective production units with rapid and high scalability, free of human pathogens, and have the ability of proper folding and assembly of complex proteins as compared to conventional fermenter-based (bacterial or yeast) and animal expression systems (Burnett & Burnett, 2020; Canter *et al*., 2005; Daniell, 2006; R. Fischer *et al*., 2004; R. Fischer & Emans, 2000; Kenter *et al*., 2009; M. Y. Khan *et al*., 2009; Lössl & Waheed, 2011; J. K. C. Ma *et al*., 2003; Meyers *et al*., 2010; Shanmugaraj *et al*., 2020; Teli & Timko, 2004; H.-H. Wang *et al*., 2009). Plants can be scaled up to the larger areas using transgenic seeds (act as stable genetic resource), thus increasing the production capacity with less costs, compared to bacterial systems where scaling up is costly due to expensive culture media and maintenance other conditions related to the fermenter-based culture (Giddings, 2001; Marsian & Lomonossoff, 2016; Rigano & Walmsley, 2005; Saba *et al*., 2020). Some other advantages are that the whole plant can be regenerated from the transformed plant cells via tissue culture with the stable inheritance of transgene in successive generations The stable transformation of plants has been done so far to provide plants resistance against

bacteria, drought, herbicides, insect, plague, salt tolerance, temperature changes, UV light, and viruses. Genetic transformation is also done to improve plant nutritional quality and increase productivity (Ashraf, 2010; Begum *et al.*, 2022; Bhat, 2010; Chandler & Brugliera, 2011; X. Chen *et al.*, 2021; Dessoky *et al.*, 2021; Dhar *et al.*, 2011; Fürst *et al.*, 2020; Godfray *et al.*, 2010; Hussain *et al.*, 2021; Kotula *et al.*, 2020; Lohani *et al.*, 2020; Martignago *et al.*, 2020; Rivera *et al.*, 2012; Schindele & Puchta, 2020; Shinwari *et al.*, 2020; Sood *et al.*, 2011; Tyagi *et al.*, 2020; Walsh, 2020). Plants were transformed to produce antibodies, an integral part of the human immune system, by integrating the respective genes in them. Furthermore, vaccines against several pathogens can be produced via plants such as against diarrhea causing bacteria. For oral vaccination, the plant's rigid and thick cell walls may protect the expressing antigen/recombinant proteins from the acidic environment of the foregut to the hindgut by encapsulating them safely and successfully in adequate amounts (Giddings, 2001; Marsian & Lomonossoff, 2016; Rigano & Walmsley, 2005; Rivera *et al.*, 2012; Saba *et al.*, 2020). In short, plant genetic engineering for the crop improvements and recombinant proteins production is a significant technology (Meyers *et al.*, 2010; Rivera *et al.*, 2012).

### 4.1.5. Genus *Nicotiana*

The Solanaceae family is an angiosperm family which includes one hundred genera and approximately 2,700 species with a great diversity of morphology, ecology, and habitat. This family contains many species which have agronomic and economic importance such as eggplant, potatoes, peppers, and tomatoes which serve as food sources. This family also contains many species of medicinal importance such as deadly nightshade, henbane, mandrake, and tobacco. Genus *Nicotiana* falls under the family Solanaceae (M. W. Chase *et al.*, 2003; Ganaie *et al.*, 2018; Olmstead *et al.*, 2008). The genus *Nicotiana* is the fifth-largest genus with eighty-two species and was established in 1753 by Carl Linnaeus. The species in the genus manifest in diverse morphological forms including small herbs, shrubs, and small trees whereas the species diversity is high in the Neotropics around the world (Elser *et al.*, 2023; Knapp, 2020; Müller-Wille & Reeds, 2007; Royal Botanic Gardens, 2019). The most studied species in the genus are *N. benthamiana*, *N. glauca*, *N. glutinosa*, *N. paniculata*, *N. rustica*, and *N. tabacum*. The species *N. rustica* and *N. tabacum* are cultivated worldwide for tobacco products.

*N. galuca* has been a spotlight for biofuel research and *N. banthaniana* is a model plant for molecular biotechnology (Elser *et al*., 2023; Pombo *et al*., 2020; Usade *et al*., 2018).

#### 4.1.5.1. *Nicotiana tabacum*

*Nicotiana tabacum* is commonly known as an economically important tobacco and model plant (Ganapathi *et al*., 2004; Y. Yang *et al*., 2022). *Nicotiana tabacum* is a natural amphidiploid, also known as allotetraploid, whose phylogenetic tree indicates an event of hybridization between two diploid progenitors, *N. sylvestris* and *N. tomentosiformis*, dating back to 4-5 million years ago. It is a perennial short-lived herbaceous plant that originated in tropical and subtropical America but is commercially grown as a cash crop around the world for cigarette production (Al-Snafi, 2022; Ganapathi *et al*., 2004; Rawat *et al*., 2013; Schiavinato *et al*., 2020; Zou *et al*., 2021).

It has many important traditional and modern uses. All parts of this plant like seeds, leaves, blooms, and roots contain different compounds that are commercially and clinically used in the whole world. Tobacco plant also has importance in ornamental industries with more than 70 species being grown as ornamental plants (Al-Lahham *et al*., 2020; Al-Snafi, 2022; Berbeć & Doroszewska, 2020).

#### 4.1.5.2. Morphology and taxonomic classification of *Nicotiana tabacum*

It is a short-lived perennial or annual non-food herbaceous little branched plant that grows up to 0.7 to 2 meters in height. The plant is moist and sticky. The plant has expanded, oblong or elliptic ovate, and thin lush green leaves (24-40 x 13-25 cm). Flowers are arranged in axillary and terminal compact corymboid panicles with pink or white. The Capsule is a little larger than the calyx in size. The capsule is oblongovoid and 20 mm long whereas the calyx is oblong and 10-12 mm long. Seeds are angled, brown, and ruminate. All parts of the plant are covered with small glandular hairs which secrete a yellowish discharge named nicotine, because of this all parts of the plant give a somewhat sticky appearance. Tobacco growth is affected by humidity, temperature and soil, best climate humidity for effective growth is 80-85%, the optimal temperature is 20-30°C and soil with normal nitrogen concentration is most suitable (Al-Snafi, 2022; T. Huang *et al*., 2021; Knapp, 2020; Leal *et al*., 2023; Rawat *et al*., 2013; Uzelac *et al*., 2021). A morphological description of *N. tabacum* is given in Figure 4.1. and its taxonomic classification is given in Figure 4.2.

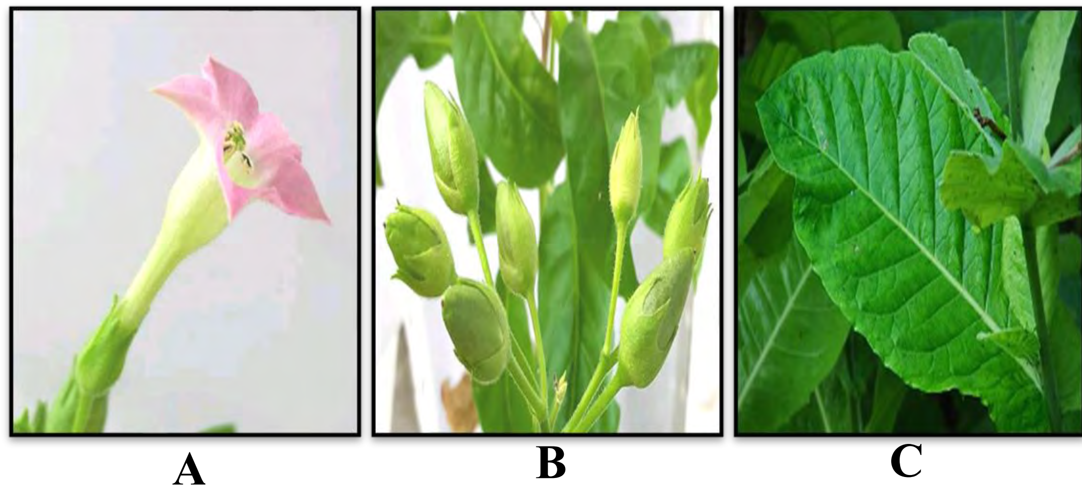



**Figure 4.1:** Morphological description of Nicotiana tabacum (A) Flower (B) Capsule (C) Leave (Adapted from Encyclopedia Britannica)

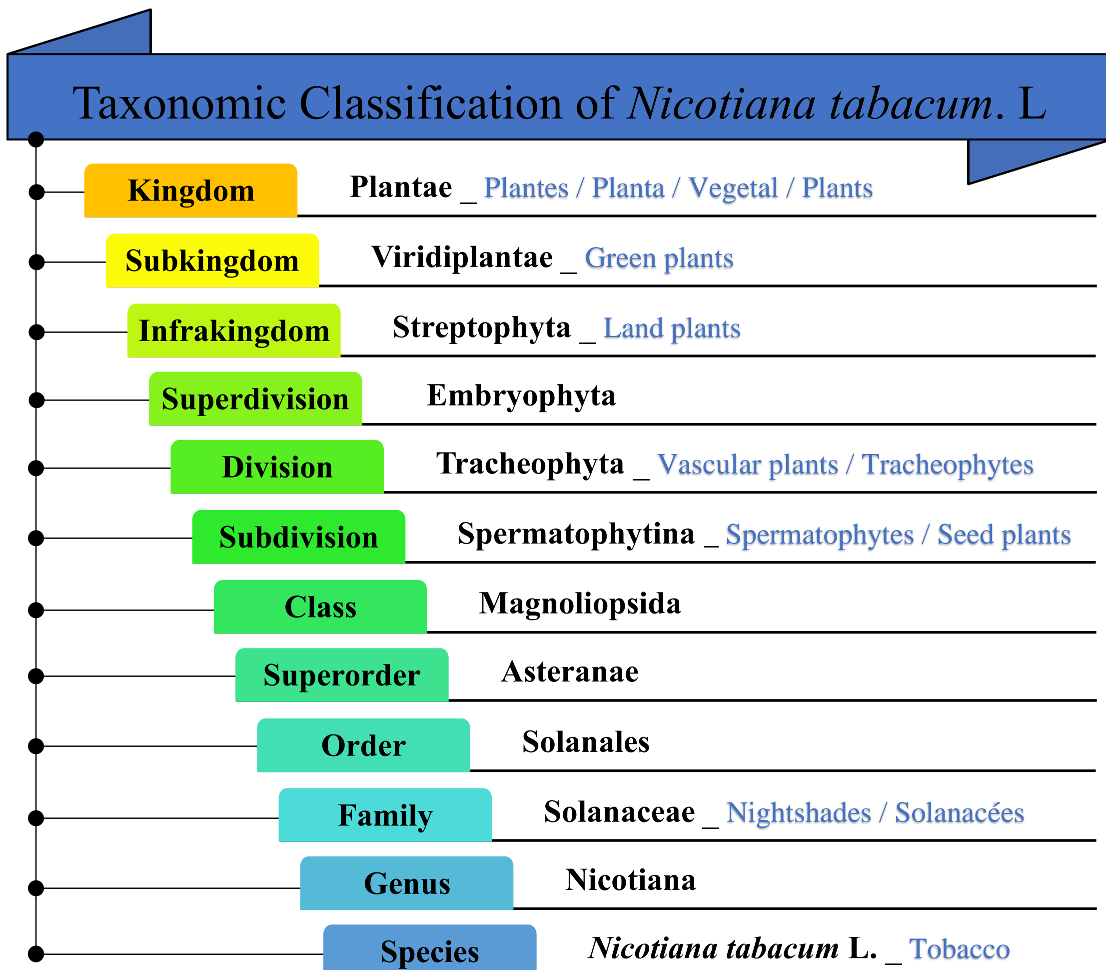



**Figure 4.2:** Taxonomic classification of *Nicotiana tabacum* L. (Al-Snafi, 2022).

#### 4.1.5.4. *Nicotiana tabacum* and biotechnology

*Nicotiana tabacum* serves as a model plant for genetic transformation due to well-characterized metabolic and genetic processes and fast growth (one hundred tons per

hectare). It is a non-food bio-factory that has great potential for the production of recombinant proteins as medicines or vaccines. The non-food plants are at low risk of contaminating plants which are sources of human food (T. Huang *et al*., 2021; Łojewska *et al*., 2020; Sheen, 1983). It was the first plant that was subjected to *Agrobacterium*-mediated transformation to produce kanamycin in 1984. Another advantage of the tobacco plant from a biotechnology point of view is its high level of totipotency, the ability of cells to divide and differentiate into tissue leading to the regeneration of the whole plant (Horsch *et al*., 1984; Łojewska *et al*., 2020; Lössl & Waheed, 2011). Other advantages such as high biomass, scalability, established tissue culturing and transformation protocols make it a model plant for the expression of a wide range of foreign transgenes. Due to these characteristics, the tobacco plant is ideal for nuclear as well as chloroplast transformation (Jube & Borthakur, 2007; Saba *et al*., 2019; Tusé *et al*., 2015).

### 4.1.6. Objectives

In the present part of the work, the objective was to stably express the OmpK antigen in tobacco nuclear genome for the development of a plant-based vaccine against vibriosis through *Agrobacterium*-mediated transformation. Further, it was aimed to check the immunogenicity of the expressed OmpK vaccine candidate in the animal models.

## 4.2. Materials and Methods

### 4.2.1. Construction of expression vector via Gateway® cloning

The *OmpK* gene having Gene bank accession no. FJ705222.1 was retrieved from NCBI. The synthesis of construct was carried out by BIOMATIK (Canada) in a pUC57 vector. The vector contained the Gateway® cloning flanking sites, *att*B1 and *att*B2 and Histidine-tag (6×His-tagged) at C-terminal to express the *OmpK* transgene in *N. tabacum*. 6xHis tag was inserted for expressed protein detection. For the construction of the binary expression vector, the Gateway® cloning strategy was used. The Gateway® cloning kit (Invitrogen, USA) was equipped with BP and LR Clonase enzymes with their corresponding buffers and the cloning reactions were executed as synthesized and depicted by Nakagawa *et al*. (2007). *OmpK* gene was cloned into pDONR™221 via BP recombination reaction to get the intermediate entry vector, pENTR-OmpK. The BP reaction was conducted by mixing the PCR amplified *att*B flanked *OmpK* gene, BP Clonase enzyme, BP Clonase reaction buffer, pDONR™221, and TE buffer by incubat-

ing the mixture for 16 hours at 25°C. Afterwards, the LR recombination reaction was carried out by mixing *att*L sites flanked pENTR-OmpK, *att*R sites flanked destination vector pGWB5, LR Clonase enzyme, LR Clonase buffer and TE buffer to get a final expression vector pEXP-OmpK by incubating the mixture for 16 hours at 25°C. Table 4.1 shows the standard protocol of BR and LR recombination reactions. The strong constitutive cauliflower mosaic virus 35S promoter controls the expression of the *OmpK* gene in the pEXP-OmpK binary vector. The proper integration of the *OmpK* gene at each step was checked by PCR with a separate set of primers. Tables 2.4 and 2.5 of Chapter 2 show the different sets, sequences and annealing temperatures of primers used to check the proper gene integration in the final binary vector.

### 4.2.2. Transformation of *E. coli* DH5α and *Agrobacterium tumefaciens*

The plasmids (pUC57 harboring the *OmpK* along with 6xHis tag, pENTR-OmpK, and pEXP-OmpK binary vector) were integrated into *E. coli* DH5α bacteria for storage (-80°C), propagation, amplification, and isolation of plasmid. The pEXP-OmpK binary vector was integrated into *Agrobacterium tumefaciens* for the transformation of the plant. For transformation, the Biorad Electroporator (USA) was used. 60 µL of freshly prepared electro-competent cells (*E. coli* DH5α or *Agrobacterium tumefaciens* strain GV3101) (in detail in section 2.2.5.1. of Chapter 2) were thawed for 10 minutes on ice and a pEXP-OmpK binary vector (2 µL) was added to it. The sample was pipetted into a cuvette. The conditions for electroporation were set according to the manufacturer's protocol and then the sample was electroporated. In the cuvette 250 µL of SOC media (preheated at room temperature) was added and gently homogenized using a pipette. Then the mixture was shifted to a 1.5 mL Eppendorf tube and incubated for 2-3 hours at 28°C or 37°C with gentle shaking for respective bacteria. Bacterial cultures were streaked on an LA plate containing respective antibiotics for the selection of transformed bacterial colonies. The plates were then placed overnight at 28°C or 37°C for the respective bacteria. Table 4.3 shows the antibiotic concentrations for the selection of bacterial colonies.

### 4.2.3. Germination of tobacco seeds

#### 4.2.3.1. Plant material

Seeds of wild-type *Nicotiana tabacum* cv. Petit Havana was obtained from the University of Natural Resources and Applied Life Sciences (BOKU), Vienna, Austria.

Seeds were stored at a cool and dry place with relative humidity (< 30%) and a temperature of 20°C.

**Table 4.1:** The standard protocol of BR and LR recombination reaction.

| **Gateway® cloning standard protocol** | |
|---|---|
| **Components** | **Concentration (µL)** |
| **BP reaction** | |
| 5X BP Clonase reaction buffer | 4 |
| *att*B PCR amplified product GoI (40-100 fmol) | 1-10 |
| BP Clonase enzyme | 4 |
| pDONR221™ (150 ng/µL) | 2 |
| TE Buffer, pH=8 | Up to 16 |
| Total volume | 20 |
| **LR reaction** | |
| 5X LR Clonase reaction buffer | 4 |
| Destination vector (150 ng/µL) | 2 |
| Entry clone (100-300 ng) | 1-10 |
| LR Clonase enzyme | 4 |
| TE Buffer, pH=8 | Up to 16 |
| Total volume | 20 |

**Table 4.2:** The antibiotic conditions for different bacterial strains.

<table>
<tr><th>Plasmid</th><th>Bacteria</th><th>Antibiotic</th><th>Concentration</th></tr>
<tr><td>pUC57_OmpK vector</td><td>E. coli DH5α</td><td>Ampicillin</td><td>100 mg/L</td></tr>
<tr><td>pENTR-OmpK vector</td><td>E. coli DH5α</td><td>Kanamycin</td><td>50 mg/L</td></tr>
<tr><td rowspan="4">pEXP-OmpK binary vector</td><td rowspan="2">E. coli DH5α</td><td>Kanamycin</td><td>50 mg/L</td></tr>
<tr><td>Tetracycline</td><td>50 mg/L</td></tr>
<tr><td rowspan="2">A. tumefaciens GV3101</td><td>Kanamycin</td><td>50 mg/L</td></tr>
<tr><td>Tetracycline</td><td>50 mg/L</td></tr>
</table>

#### 4.2.3.2. Seed sterilization

The *N. tabacum* cv. Petit Havana (wild type) seeds were surface sterilized for 1 minute with 70% (v/v) ethanol solution and rinsed with double distilled autoclave water thrice. Then dried on filter paper in a Laminar Flow Hood (LFH).

#### 4.2.3.3. Seed germination

Sterilized seeds were *in vitro* grown on agar-solidified Murashige and Skoog (1962) medium containing 30 g/L sucrose in parafilm-sealed jars. Seeds were grown under optimum conditions (16 hours light/8 hours dark; 25±1°C) in a growth room.

### 4.2.4. Optimization of hygromycin

To determine the inhibitory concentration of hygromycin which halts the growth of explants, different concentrations of hygromycin were used for optimization for tobacco plants. The explants were placed on RMOP media containing agar (7.0 gm/L), BAP (1.0 mg/L), Myo-inositol (100 mg/L), NAA (0.1 mg/L), sucrose (30 gm/L), thiamine-HCl (1.0 mg/L) and different concentrations of hygromycin (0, 20, 30, and 50 mg/L). In each selection media, explants were inoculated for four weeks at 25±1°C with a 16-hour photoperiod. Three replications and five explants were used per treatment.

### 4.2.5. *Agrobacterium*-mediated transformation of tobacco

Transformation of *tobacco* was done by using the GV3101 strain of *Agrobacterium* harboring binary expression vector pEXP-OmpK.

#### 4.2.5.1. Preparation of explant

Leaves of *in vitro* grown 2-3 weeks old tobacco plants were sliced into pieces measuring 5-8 mm. The sliced leaves were positioned horizontally on the surface of RMOP media on glass petri plates and then sealed with parafilm.

#### 4.2.5.2. Media preparation for transformation

The composition of different media used for the transformation of tobacco in Annexure 4.1. The media used in the transformation of tobacco explants were co-cultivation media, washing media, and selection media.

Co-cultivation media for leaves was prepared by adding acetosyringone in RMOP media. Media was poured into sterile petri plates sealed by parafilm and stored in the growth room until further use.

The washing media was liquid MS media. It was prepared in distilled water, pH was adjusted, and sterilized in the autoclave. Cefotaxime was added before use.

Selection media for leaves was prepared by adding hygromycin, cefotaxime and hormones in MS media poured into sterile plates and left to solidify. Then plates were sealed through parafilm and kept in the growth room until further use.

#### 4.2.5.3. Transformation of tobacco plants

The following steps were followed for the transformation of tobacco plants via *Agrobacterium tumefaciens*. The first step was the co-cultivation of tobacco explants with *A. tumefaciens*. For this purpose, *A. tumefaciens* containing pEXP-OmpK bacterial culture ($OD_{600}$ 0.6-0.8) was pellet down via centrifugation for 10 minutes at 3600g and then re-suspended in liquid MS media containing acetosyringone (infection media). Then, leave pieces, sliced to a size of 5-8 mm, from the 10-15 days old tobacco plants grown *in vitro* were infected with *Agrobacterium*-infection medium for eight minutes. After the infection, the explants were positioned horizontally on the co-cultivation medium surface. Co-cultivation media with infected explants were kept in the growth room for two days at 25±2°C in the dark.

In the second step, the selection of transformed explants on appropriate antibiotics was done. After completion of a specific co-cultivation period for two days, explants were washed for 5 minutes by dipping in washing media. Then explants were blotted on sterilized filter paper to dry. After complete drying, for the selection of transformed plants, the explants were shifted to selection media containing 30 mg/L hygromycin (Pathi *et al*., 2013). *In vitro* transgenic plants were grown under optimum conditions (16 hours light/8 hours dark; 25±1°C) in a growth room. After every two weeks washing was done, and the selection media was changed. Transgenic shoots were then shifted to jars on MS medium containing antibiotics for rooting. The transgenic plants with established roots were subsequently transferred to pots and moved to the greenhouse for acclimatization.

### 4.2.6. Molecular analyses

#### 4.2.6.1. Confirmation of transgene integration by PCR

To confirm the *OmpK* gene in the transformed bacterial colonies and transgenic *N. tabacum* PCR was performed. The method for isolation of bacterial plasmid is described in detail in Chapter 2 section 2.2.6.1. The genomic DNA from the wild-type

and transgenic tobacco plants (100 mg leaf material) was extracted via hexadecyltrimethylammonium bromide (CTAB) proposed by Murray and Thompson (Murray & Thompson, 1980). The details protocol has been described in Chapter 2 section 2.2.6.2. PCR was performed by using ThermoFisher Scientific (USA) Taq polymerase standard PCR Kit. 1 µM of both primers (forward and reverse) and 100 ng of template DNA was used. Integration of *OmpK* transgene within transgenic bacteria and the genomic DNA of tobacco was confirmed by different sets of primers mentioned in Table 2.5 of Chapter 2. The PCR conditions, annealing temperatures and expected fragment size are given in Tables 2.5, 2.9, and 2.10 of chapter 2. The PCR product was resolved on 1% agarose gel stained with 5 ng/mL ethidium bromide. The bands were observed using a gel documentation system (FluorChem FC3, USA).

#### 4.2.6.2. Copy number determination via qRT-PCR

Transgene copy number in the transformed plant was determined through quantitative real-time PCR (MyGo Pro Stokesley, UK) according to the protocol described by Wen *et al*. (2012). The composition of the qRT-PCR reaction mixture is given in Table 4.3. 20 µL qRT-PCR reaction mixture contained 10 µL SYBR Green (ThermoFisher Scientific, USA), 1 µL of 100 ng template DNA, 1 µM each of forward and reverse primer, and 8 µL of autoclaved distilled water. By using the genomic DNA from the wild-type *N. tabacum* and transgenic lines, the standard curves were determined for both *OmpK* transgene and the endogenous *β*-actin gene. DNA was diluted in series to get 1, 10, 100, and 1000 ng as final concentrations per reaction. Subsequently, qRT-PCR was run with independent repetitions in triplicate. A linear correlation between the input DNA amount and fluorescence level was established. The qRT-PCR amplification conditions are given in Table 4.3.

#### 4.2.6.3. Protein extraction from tobacco leaves

Leaf material from transgenic and wild-type tobacco (100 mg) was grounded in liquid nitrogen and then re-suspended in a 400 µL of protein extraction buffer (Annexure 4.2) to extract total soluble protein (TSP). The homogenized sample was centrifuged at 20 × 1000 g at 4ºC for 10 minutes. The supernatant was collected and was regarded as a soluble fraction of protein. Total protein concentration in the soluble fraction was determined via Bradford assay against bovine serum albumin (BSA) as standard (discussed in detail in Chapter 2).

**Table 4.3:** Master mixture composition and conditions for qRT-PCR

| **Master mixture for qRT-PCR** | | | | |
|---|---|---|---|---|
| **Components** | | **20 µl reaction** | | |
| Forward primer | | 0.5 µl | | |
| Reverse primer | | 0.5 µl | | |
| SYBR Green | | 10 µl | | |
| Template DNA (~100 ng) | | 1 µl | | |
| Water | | 8 µl | | |
| **Conditions for qRT-PCR** | | | | |
| **Programs name** | | **Temp. (˚C)** | **Ramp (˚C/s)** | **Hold (s)** |
| Hold | | 95 | 4 | 600 |
| Amplification (Three steps) | Denaturation | 95 | 5 | 10 |
| | Annealing | 57 | 4 | 45 |
| | Extension | 72 | 5 | 15 |
| Pre-melt hold | | 95 | 5 | 15 |
| High resolution Melting | Initial stage | 57 | 4 | 60 |
| | Final stage | 95 | 0.05 | 15 |

#### 4.2.6.4. Western blot analysis

The protocol of Western blotting has been described in detail in Chapter 2 section 2.2.11.

#### 4.2.6.5. Quantification of transgenic protein via ELISA

Protein extracts were prepared in extraction buffer (Annexure 2.9 of Chapter 2) from the fresh leaf tissue of transgenic tobacco. The homogenized sample was centrifuged at 20,000 g at 4°C for 10 minutes and the supernatant (SF) was collected. This supernatant was further used for the transgenic protein (TP) quantification via ELISA by following the protocol given in detail in section 2.2.13 of Chapter 2. The amount of TP as a percentage of the total soluble protein (TSP) of transformed leaf material was calculated by the formula:

$$\% \boldsymbol{TSP} = \left(\frac{\boldsymbol{TP}}{\boldsymbol{TSP}}\right) \times \mathbf{100.}$$

### 4.2.7. Pre-clinical trials in mice and fish

The experiment on the mice was conducted at the Biological Sciences' Primate facility located at Quaid-i-Azam University, Islamabad, Pakistan, and the experiment on the fish was carried out in the Lab. The standard protocols outlined and approved by ethical committee of Quaid-i-Azam University Islamabad and published in the "Guide for the Care and Use of Laboratory Animal Resources" by the National Academies Press (Washington D.C.) were followed during all animal handling.

Adult 8-10 weeks BALB/c mice were divided into seven groups (six members each), labelled as Group A: Normal group with no dose; Group B: PBS solution oral delivery; Group C: PBS solution subcutaneous delivery; Group D: WT tobacco TSP oral delivery; Group E: WT tobacco TSP subcutaneous delivery; Group F: Transgenic tobacco TSP oral delivery, and Group G: Transgenic tobacco TSP subcutaneous delivery. For oral and subcutaneous dosing of mice, the dose was calculated by rearranging the formula given by Verma *et al*. (2008) so that Group F and G would receive 10 µg of transgenic protein by dissolving an appropriate amount of transgenic plant in 500 µL of PBS for oral dosing and 100 µL of PBS via subcutaneous injections.

$$\boldsymbol{Amount\ of\ transgenic\ protein = \frac{TP \times V_{PBS}}{W_{TLM} \times 10^6}}$$

TP = Transgenic protein (ng/mL or ng/gm), $V_{PBS/pellet}$ = PBS/pellet volume (mL or gm), and $W_{TLM}$ = Transformed leaf material (grams). The mice were immunized on the 1st, 7th, 14th, and 21st day. To isolate serum from the blood of mice, they were euthanized after one week of the last boost.

For the immunogenicity assay, the fish model *Hypophthalmichthys molitrix* (silver carp; 57.8±1.3g and 19.4±1.1cm) was used. Fish were divided into three groups (ten members each) labelled as Group H: Normal group on commercial feed; Group I: WT tobacco oral delivery mixed in feed pellet, and Group J: Transgenic tobacco oral delivery mixed in feed pellet. For oral dosing of fish, the protocol described by Shin *et al*. (2013) was followed, with little modification in the dosing pattern, so that each fish could receive 10 µg/10gm fish body weight of transgenic protein in a feed pellet. Fish immunization was done on the 1st, 5th, 10th, and 15th days. Fish blood samples were collected after seven days of the last immunization.

ELISA (enzyme-linked immunosorbent assay) was performed to assess the blood serum IgG level of mice and fish blood. Transgenic tobacco TSP (5 µg/well) for 24 hours at 4°C was coated in a 96-well microtiter plate. The plate was washed three times with TBS-T (TBS containing 0.1% Tween-20) followed by blocking with TBS-TM (TBS containing 0.3% Tween-20 and 3% skimmed milk) for 1 hour at 37°C. 50 µL of isolated test sera was loaded into each well followed by addition of 50 µL of 1:10,000 dilution of HRP-conjugated goat anti-mouse IgG secondary antibody. Then for 1 hour the incubation of the plate was done at 37°C and washed with TBS-T thrice. 100 µL 3,3´,5,5´-tetramethylbenzidine (TMB) substrate (ThermoFisher Scientific, USA) was added to each well. The reaction was stopped by 0.16 M $H_2SO_4$ (100 µL) after 10-20 minutes. Absorbance was recorded at 450 nm with a microtiter plate reader (Bio-Rad, Japan).

### 4.2.8. Statistical analysis

To perform statistical analysis such as for determining the standard deviations, and drawing graphs Microsoft Excel was used.

## 4.3. Results

### 4.3.1. Final expression vector

The final transformation binary vector pEXP-OmpK was constructed via Gateway® cloning for the transformation of tobacco. The schematic representation of BP and LR reaction steps is shown in Figure 4.3, whereas the final transformation binary vector pEXP-OmpK is shown in Figure 4.4. The pEXP-OmpK consisted of *OmpK* transgene from *V. anguillarum* with a 6×His-tag attached at the C-terminal, *neomycin phosphotransferase* II (*npt*II) gene (kanamycin) for the selection of transformed bacterial colonies, and *hygromycin phosphotransferase* (*hpt*) gene (hygromycin) for the selection of transformed plants. Figure 4.5(A) shows the PCR-amplified product used in the BP reaction. The proper integration of the *OmpK* gene in the entry clone pENTR-OmpK was confirmed with PCR by using primer set “A” (Table 2.5 of Chapter 2). The 500 bp band presence on agarose gel confirmed the successful integration of the *OmpK* gene within the intermediate entry clone. The final expression pEXP-OmpK binary vector was confirmed with PCR by using primer set “C” (Table 2.5 of Chapter 2). The 870 bp band on agarose gel affirmed the successful integration of the *OmpK* gene in

the final pEXP-OmpK binary vector. Figure 4.5. A and B show the PCR confirmation of the intermediate entry clone and final expression vector harboring the OmpK gene.

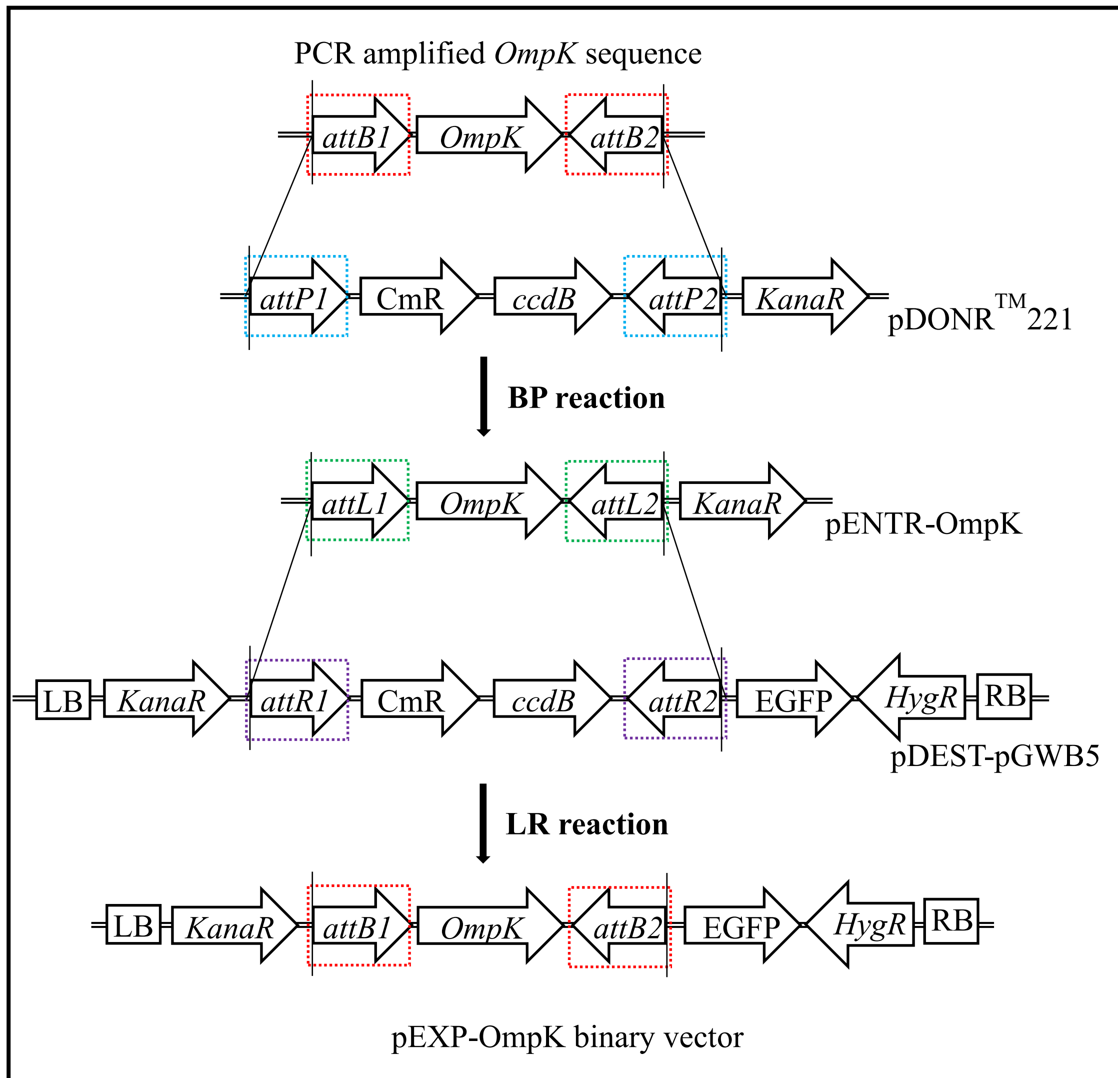



**Figure 4.3: Schematic representation of Gateway® cloning to get pExp-OmpK binary vector**. attB1/B2/L1/L2/R1/R2: Gateway® cloning recombination sites; *ccdB*: control of cell death gene; CmR: chloramphenicol resistance gene; EGFP: enhanced GFP; *HygR*: gene conferring resistance to hygromycin; *KanaR*: gene conferring resistance to kanamycin; LB: left border; *OmpK*: *OmpK* gene from *V. anguillarum*; RB: right border.

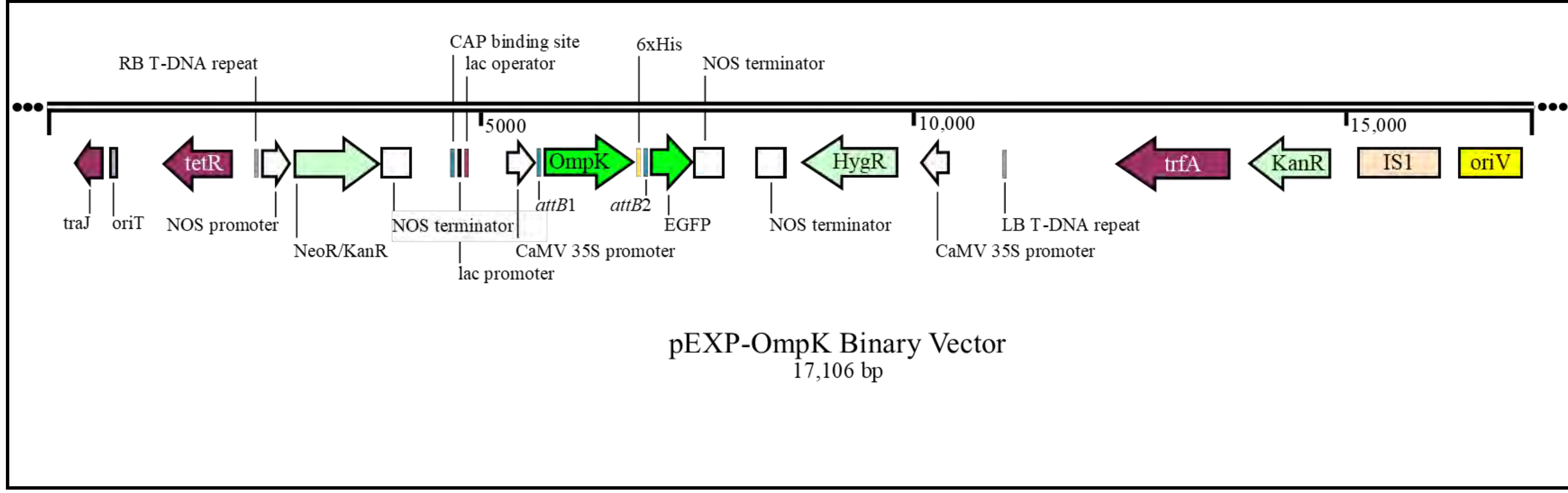



**Figure 4.4: Schematic representation of pEXP-OmpK binary vector showing expression cassette along with pDEST-pGWB5 backbone.** attB1/B2: Gateway® recombination sites; CaMV 35S Promoter: strong constitutive promoter from cauliflower mosaic virus; CAP binding site: *E. coli* catabolite activator protein; *EGFP*: the original enhanced GFP; *HygR*: a gene conferring resistance to hygromycin; IS1: insertion sequence 1; lac promotor: promoter for the *E. coli* lac operon; lac operator: lac repressor encoded by lacI; LB T-DNA repeat: left border repeat from nopaline C58 T-DNA; *NeoR/KanR*: a gene conferring resistance to kanamycin; NOS promotor: nopaline synthase promoter; NOS terminator: nopaline synthase terminator and poly(A) signal; *OmpK*: *OmpK* gene from *V. anguillarum*; oriT: incP origin of transfer; OriV: incP origin of replication. RB T-DNA repeat: right border repeat from nopaline C58 T-DNA; tetR: tetracycline resistance regulatory protein; trfA: trans-acting replication protein that binds to and activates oriV; traJ: oriT-recognizing protein; 6xHis: 6xHis affinity tag

### 4.3.2. Transformation of *E. coli* DH5α and *Agrobacterium tumefaciens*

The plasmids (pUC57 harboring the *OmpK* along with 6xHis tag, pENTR-OmpK, and pEXP-OmpK binary vector) for storage, propagation, amplification, and isolation of plasmid were integrated into *E. coli* DH5α bacteria The successful transformation of *E. coli* DH5α with pUC57 harboring the *OmpK* along with 6xHis tag, pENTR-OmpK, and pEXP-OmpK binary vector and transformation of *A. tumefaciens* with pEXP-OmpK binary vector on the respective antibiotics is shown in figure 4.6. The PCR-based confirmation of the successful transformation of *E. coli* DH5α with pUC57 harboring the *OmpK* along with 6xHis tag, pENTR-OmpK, and pEXP-OmpK binary vector is shown in Figures 4.7 and 4.8. Figure 4.9 shows PCR confirmation of correct integration and successful transformation of the pEXP-OmpK final expression in *A. tumefaciens*.

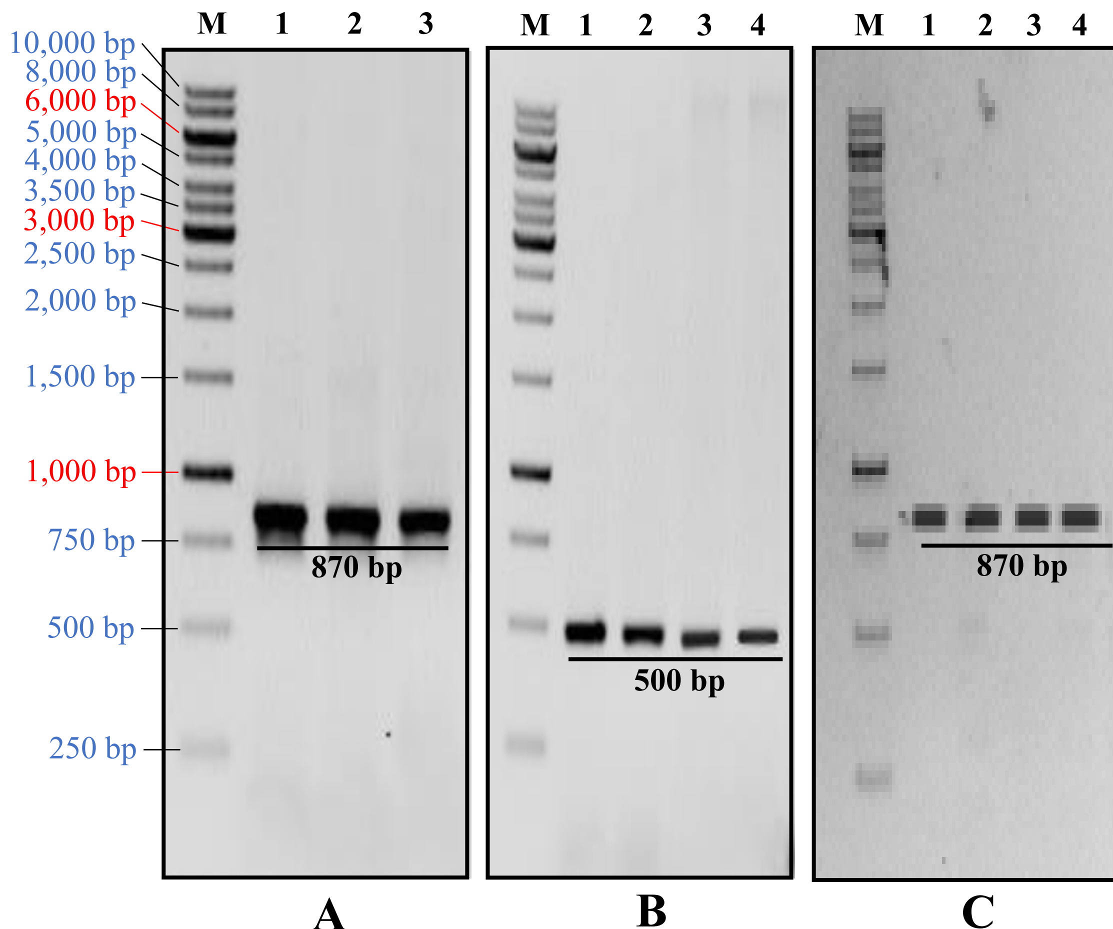



**Figure 4.5: PCR-based confirmation of Gateway® Cloning. (A)** Amplified OmpK PCR product used in BP reaction **(B)** Confirmation of proper integration of OmpK gene intermediate entry clone **(C)** Confirmation of proper integration of OmpK gene in the final expression vector. M: 1Kb Marker; 1, 2, 3, 4: PCR replicates.

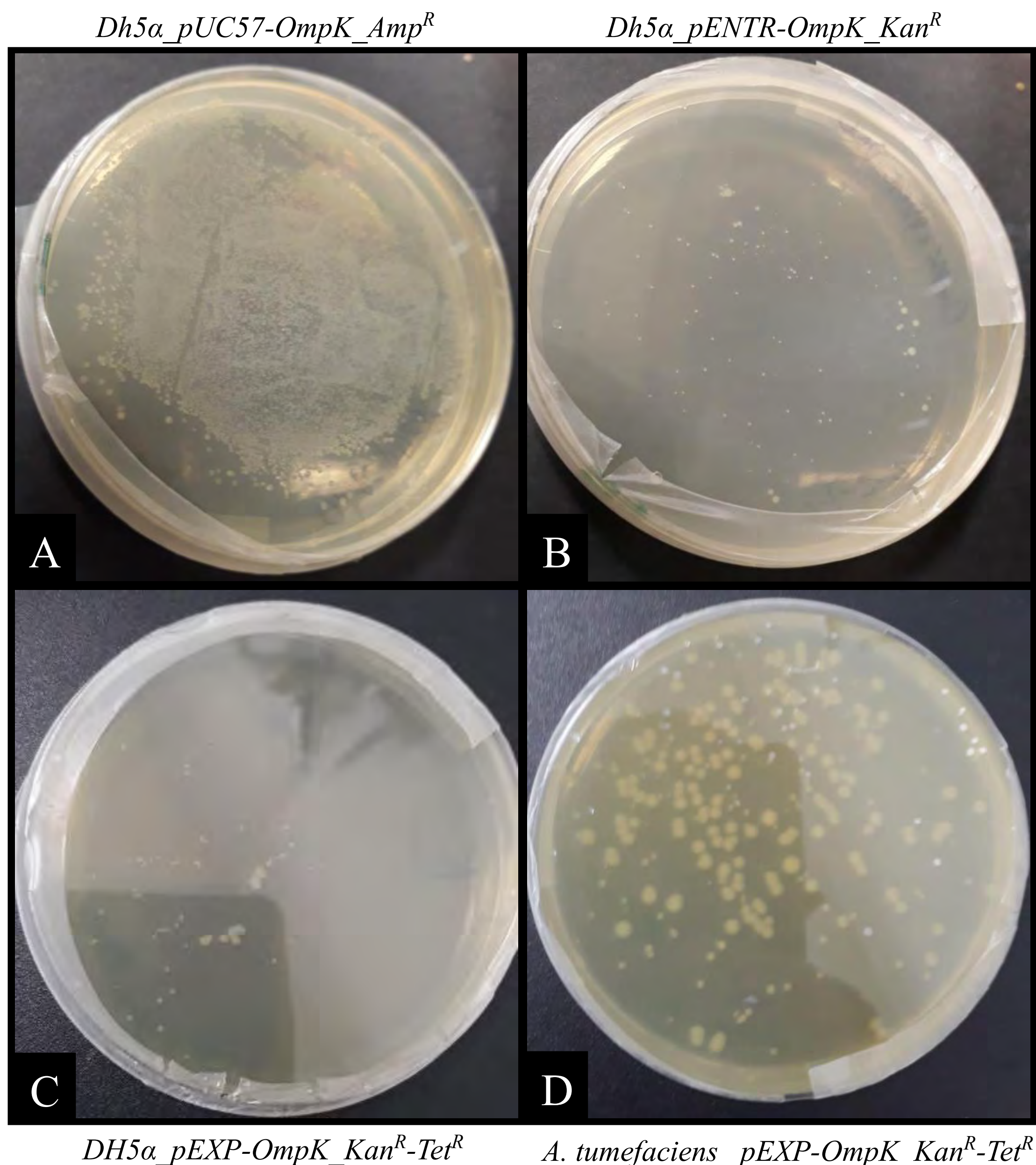



**Figure 4.6: Agar plates representing transformed bacterial colonies (A)** DH5α harboring pUC57-OmpK plasmid **(B)** DH5α harboring pENTR-OmpK entry clone **(C)** DH5α harboring pEXP-OmpK final expression vector (D) *A. tumefaciens* harboring pEXP-OmpK final expression vector.

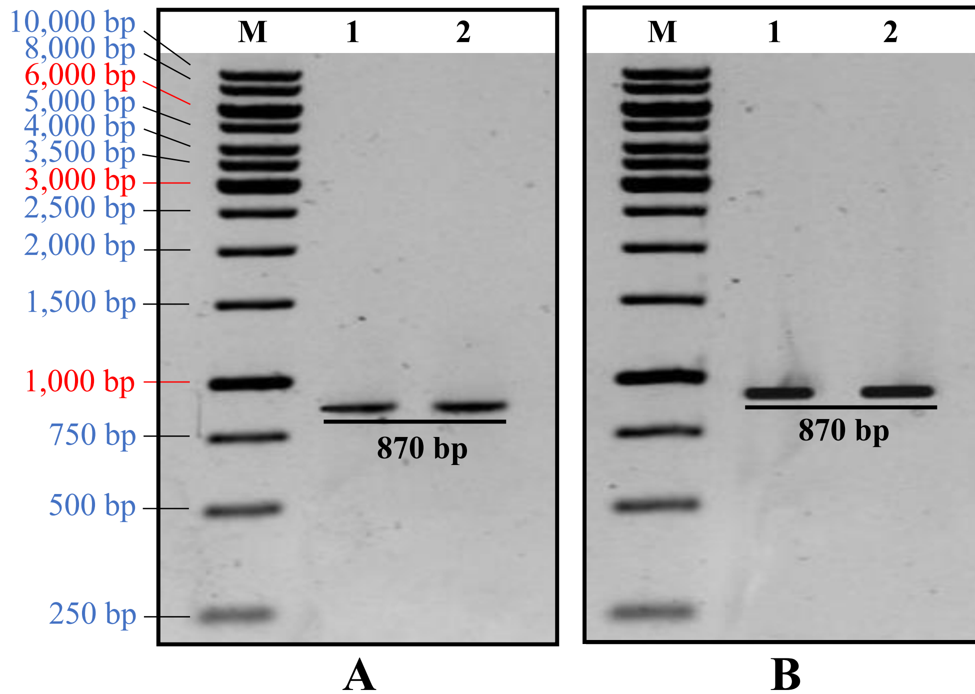



**Figure 4.7: PCR confirmation of agar plates representing transformed bacterial colonies (A)** DH5α harboring pUC57-OmpK plasmid **(B)** DH5α harboring pENTR-OmpK entry clone. P1-P2: isolated plasmid of two independent bacterial colonies

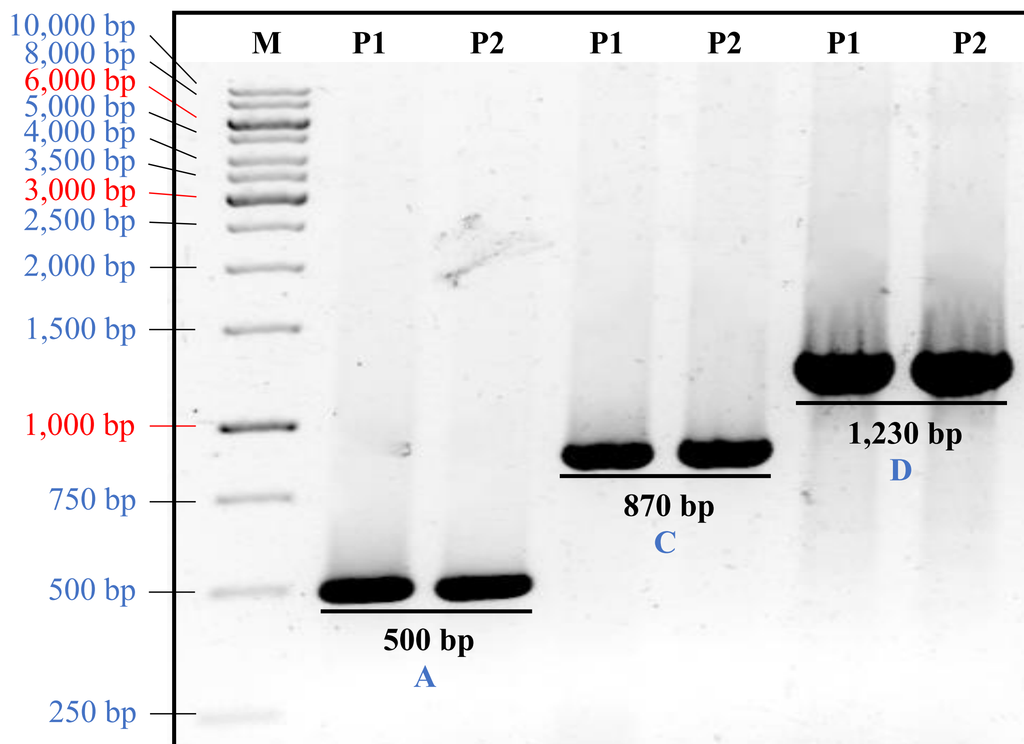



**Figure 4.8: PCR confirmation of DH5α harboring pEXP-OmpK final expression vector.** P1-P2: isolated plasmid of two independent bacterial colonies. A, C, D: different sets of primers.

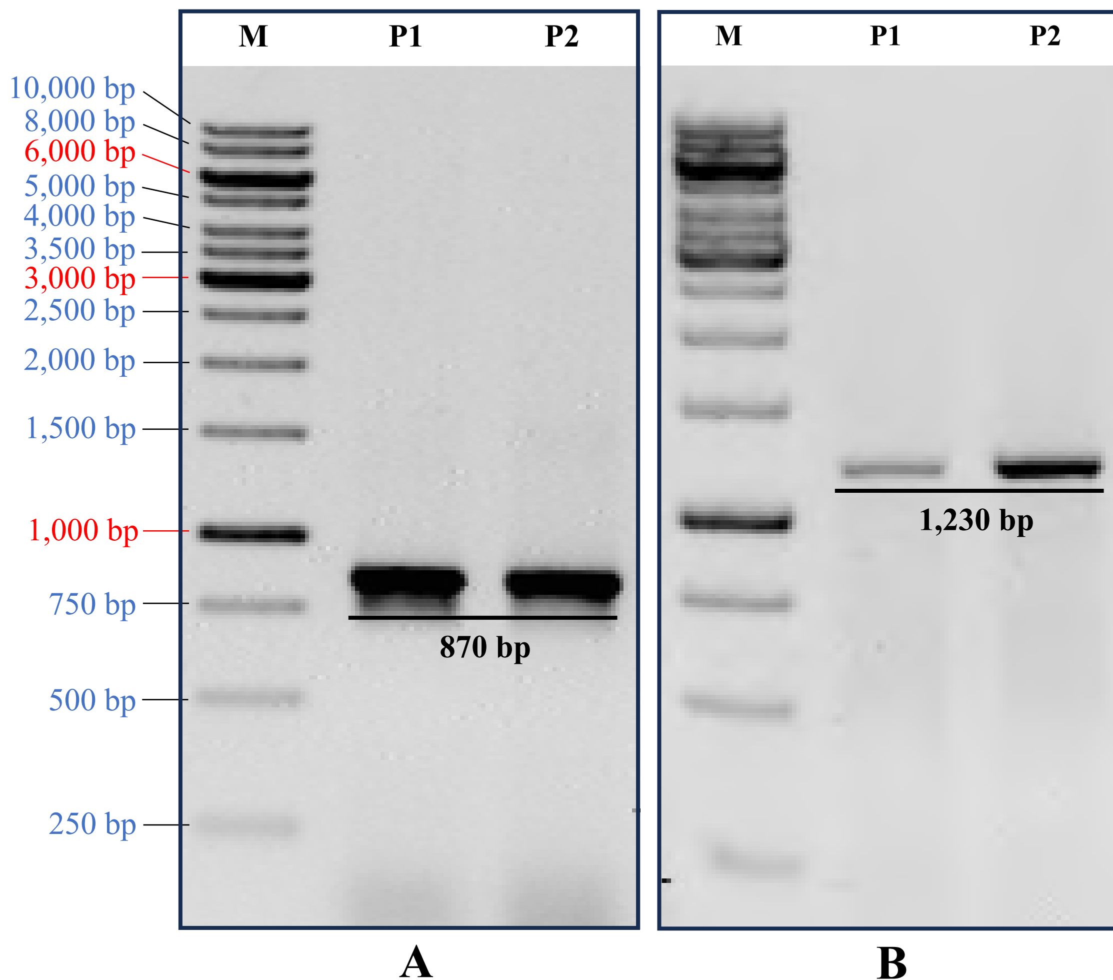



**Figure 4.9: PCR confirmation *A. tumefaciens* harboring pEXP-OmpK final expression vector. (A)** PCR confirmation with primer set "C" **(B)** PCR confirmation with primer set "D". P1-P2: isolated plasmid of two independent bacterial colonies.

### 4.3.3. Effect of hygromycin on shoot regeneration

The antibiotic optimal amount for plant transformation and growth was determined by placing explants on hygromycin at different concentrations. After thirty days of culture, 85% of the explant was regeneration into shoots when placed on a hygromycin-lacking RMOP medium. In the case of media containing 20 mg/L hygromycin the shoot regeneration was 50%. The explants cultured on the 30 mg/L hygromycin became pale within one week 90% of explants were bleached and died out and no shoot was regenerated within 30 days. Whereas 50 mg/L hygromycin concentration completely inhibited callus and shoot induction (Figure 4.10). Hence, 30 mg/L hygromycin was used for the selection of transformed shoots from explants.

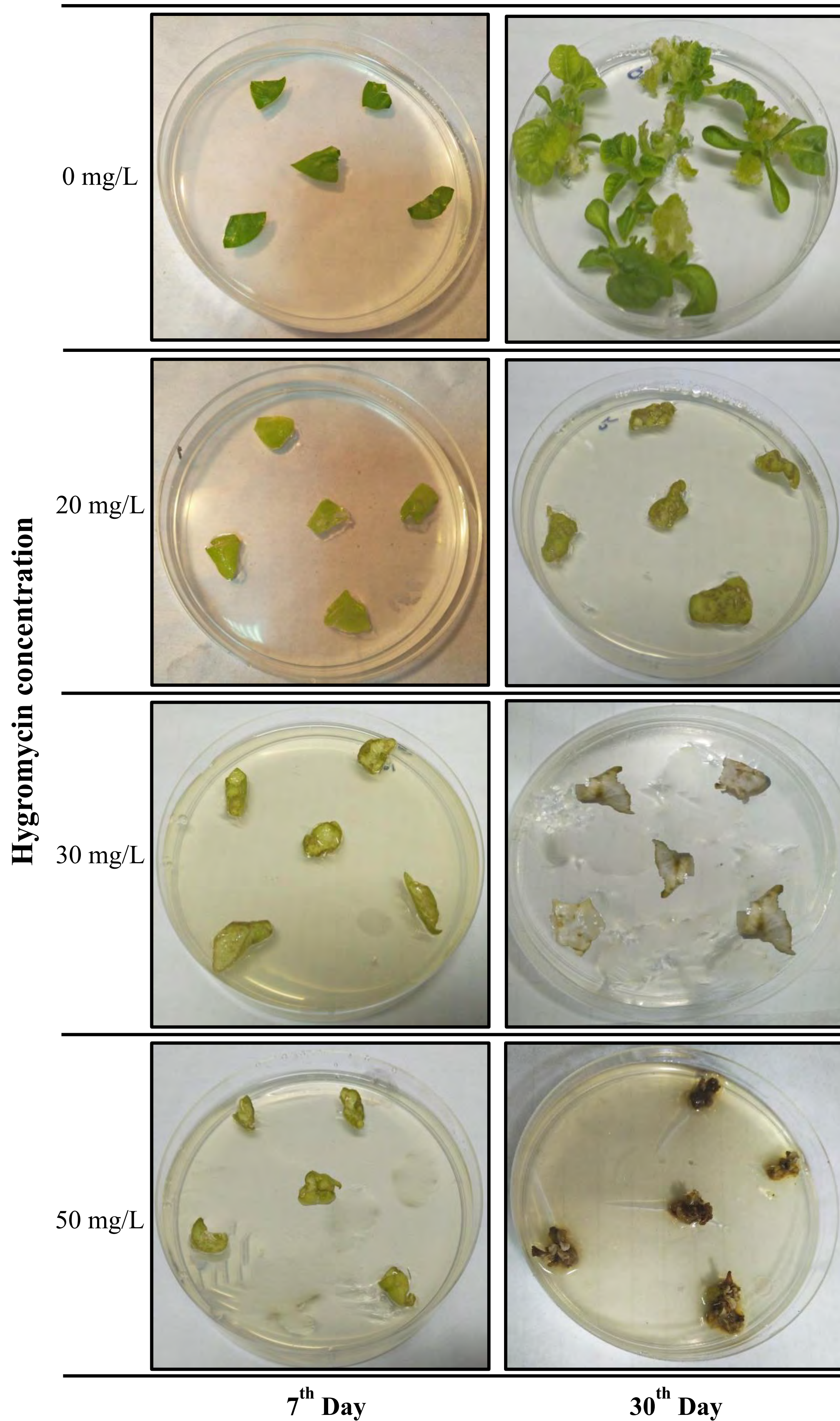



**Figure 4.10:** Optimization of hygromycin for explants.

### 4.3.4. Transgenic tobacco plant regeneration

The tobacco explants after infection for 8-10 minutes with *A. tumefaciens* harboring pEXP-OmpK were placed on a co-cultivation medium for two days followed by shifting to hygromycin (30 mg/L) supplemented RMOP medium. On the selection medium, the untransformed plants started bleaching after 10 days while in transformed explants green shoots appeared. The regenerating shoots were transferred to a fresh hygromycin-supplemented RMOP medium in glass jars. Transformed tobacco plants were then acclimatized to the soil. The shoots regeneration on the selection medium indicated that the plants were putatively transformed, which was subsequently affirmed by PCR. Figures 4.11 and 4.12 show independent transgenic lines regeneration.

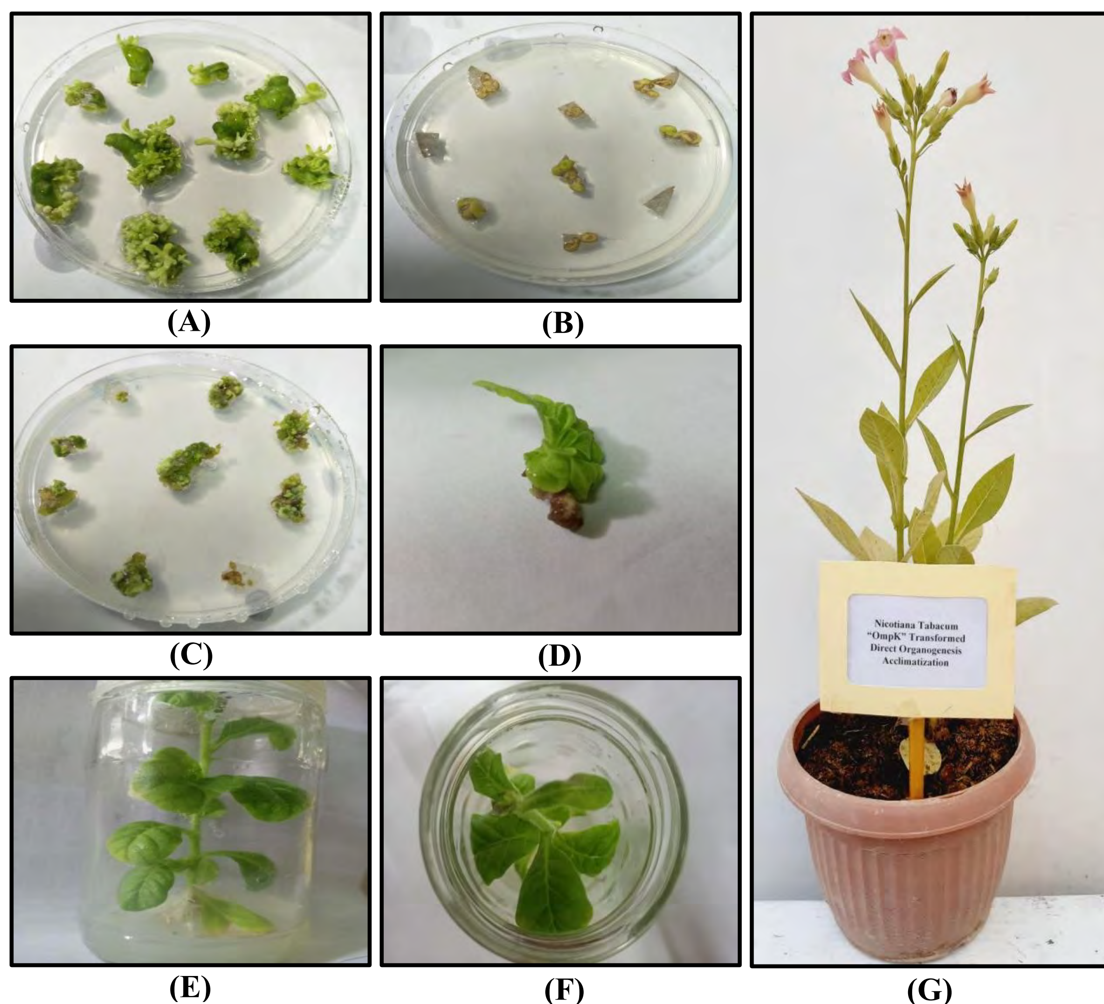



**Figure 4.11: OmpK L1 transgenic line regeneration. (A)** WT tobacco explants on an RMOP medium. **(B)** Untransformed explant on selection medium. **(C)** OmpK transformed plant on selection media. **(D)** Transformed explants shooting on selection medium **(E & F)** Transgenic plant rooting. **(G)** Transgenic plant acclimatization.

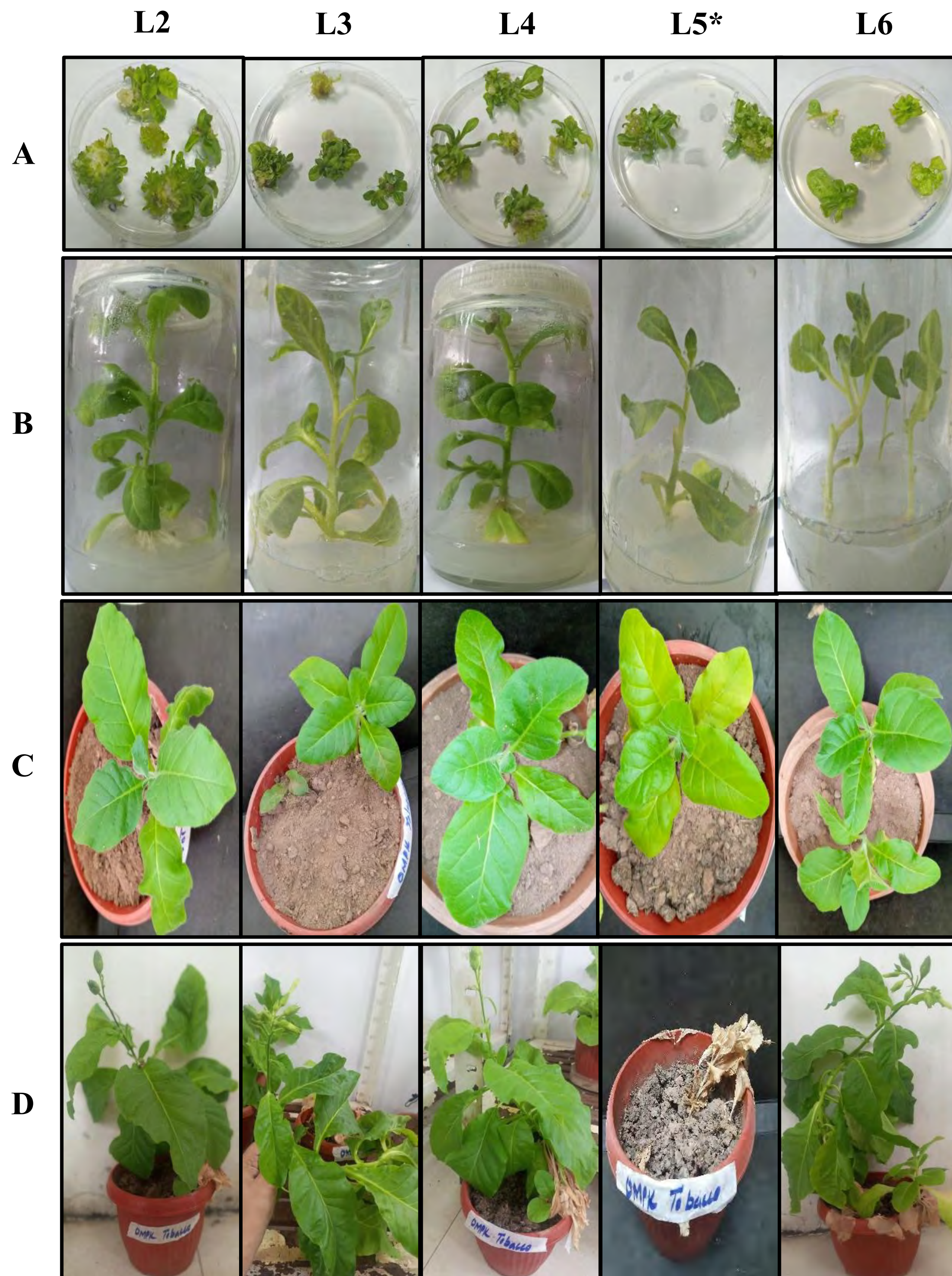



**Figure 4.12: OmpK transformed tobacco regeneration on 30 mg/L hygromycin-supplemented RPOM medium and soil. (A)** Shooting of transgenic explants **(B)** Rooting of transgenic explants **(C)** Acclimatization of transgenic shoots in soil **(D)** Flowering and seeding of transformed plants. L2-L6: Independent OmpK transgenic tobacco lines. *: The L5 transgenic line could not reach flowering and died early

### 4.3.5. Confirmation of *OmpK* transgene via PCR

To affirm the integration of the *OmpK* gene harboring expression cassette within the nuclear genome of presumed transformed plants, the PCR was performed using different sets of primers. The expected bands were obtained in transgenic lines confirming the *OmpK* gene integration within the tobacco nuclear genome. Figure 4.13 shows amplified PCR products with primer set "E" for all six transgenic lines on agarose gel with no band observed for wild-type plant. Further confirmation of L1 and L2 transgenic lines was done with different sets of primers (Figure 4.14).

### 4.3.6. Copy number determination via qRT-PCR

qRT-PCR was conducted on transgenic lines confirmed by PCR, relative to the endogenous $\beta$-actin gene, to determine the *OmpK* gene copy number. By following Wen *et al*. (2012) for the *OmpK* and $\beta$-actin gene (control), standard curves were obtained by using serial dilution with slopes values of -1.289 and -1.234, and correlation coefficients values of 0.995 and 0.943, respectively. From the standard curve of the *OmpK* gene ($SQ_{trans}$) and $\beta$-actin gene ($SQ_{end}$) the starting quantities were evaluated, and the transgene (*OmpK*) copy number was determined by using the following formula:

$$\boldsymbol{\delta rline} = \boldsymbol{rline}[(\boldsymbol{\delta SQ_{trans}}/\boldsymbol{SQ_{trans}})^2 + (\boldsymbol{\delta SQ_{end}}/\boldsymbol{SQ_{end}})^2]^{1/2}$$

A maximum of two copies of the integrated *OmpK* gene were integrated into OmpK transgenic lines L1-5 and one copy in line L6 of OmpK transgenic tobacco line.

### 4.3.7. OmpK protein confirmation and quantification

Western blot was done to confirm the OmpK protein expression using an anti-His antibody. The OmpK protein molecular weight along with His-tag was anticipated to be 30.21 kDa (monomeric form). A clear band of transgenic protein extracted from the leaves of transgenic plants in monomeric form was visible in all independent transgenic lines of tobacco plants (Figure 3.15). No band was detected in the WT tobacco plants. Ponceau staining of blot also confirmed the OmpK protein in all transgenic lines. Quantification of transgenic protein in all transgenic lines (L1-L6) was conducted via ELISA to determine which line exhibited higher expression level of OmpK by following Verma *et al*. (2008). For this purpose, a series of dilution of total soluble protein isolated from transformed and wild-type tobacco leaves was prepared. The

estimation of OmpK protein in transgenic plants was carried out by comparing it dilution series of OmpK protein ordered from the company. The OmpK protein maximum expression calculated was 0.38% of TSP in transgenic line L1 (Figure 4.16).

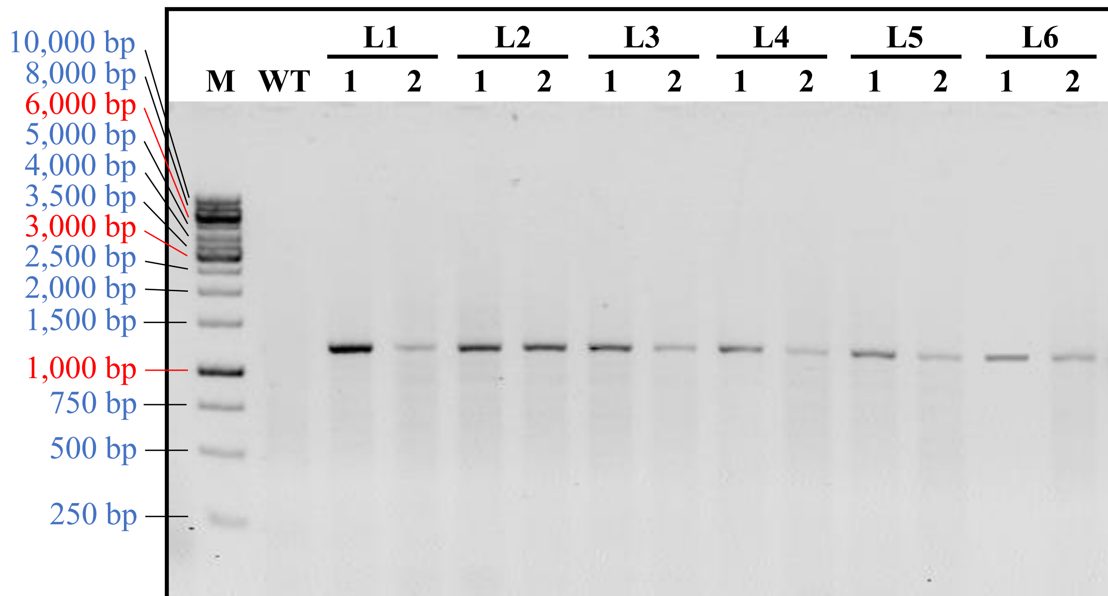



**Figure 4.13: *OmpK* gene integration confirmation in all transgenic lines of tobacco.** M: 1 kb DNA ladder; 1, 2: PCR replicates; WT: wild-type plant; L1-L6: Independent OmpK transgenic tobacco lines.

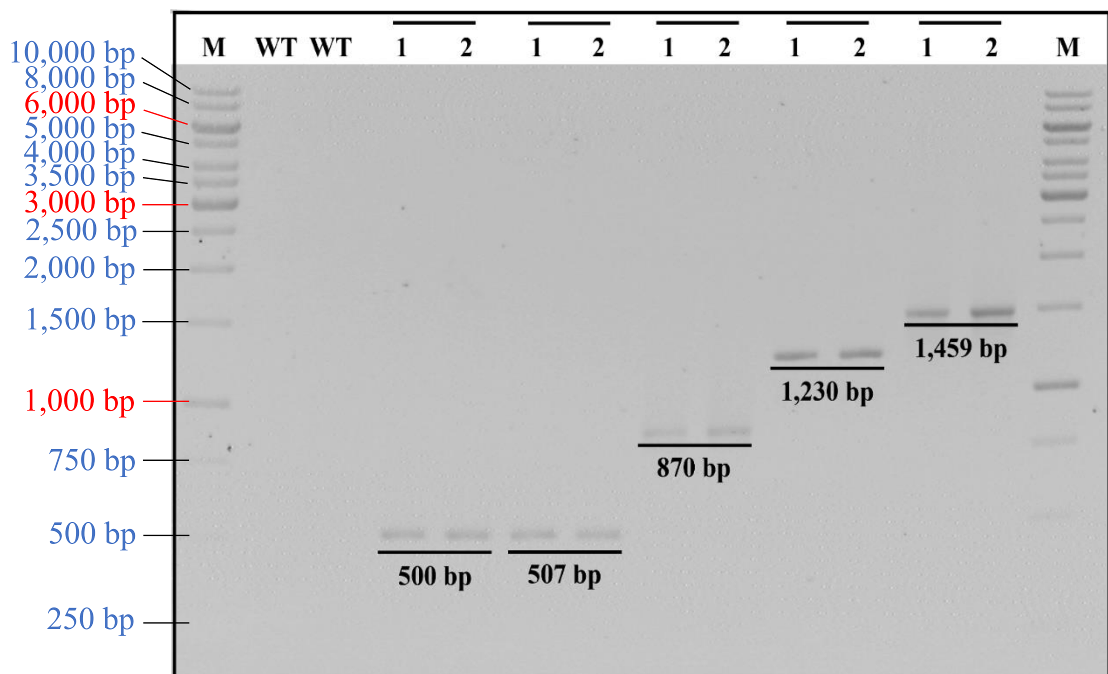



**Figure 4.14: *OmpK* gene integration confirmation in L1 and L2 transgenic line of tobacco.** M: 1 kb DNA ladder; 1, 2: two OmpK independent transgenic lines; WT: wild-type plant; The bar line represents a separate set of primers.

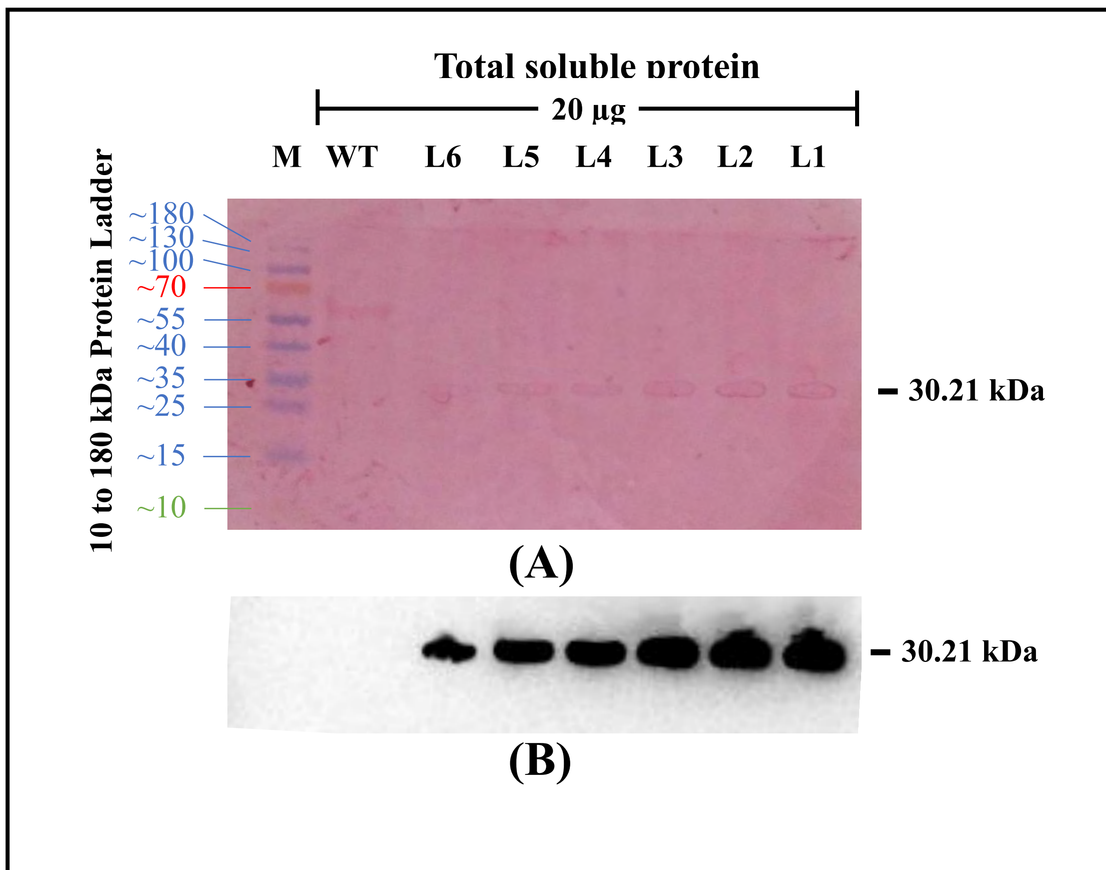



**Figure 4.15: OmpK protein conformation extracted from transformed tobacco plants (A)** Ponceau Stanning **(B)** Western blotting. L1-6: six independently generated OmpK transgenic tobacco lines; WT: wild-type plant; M: 10-180 kDa protein marker.

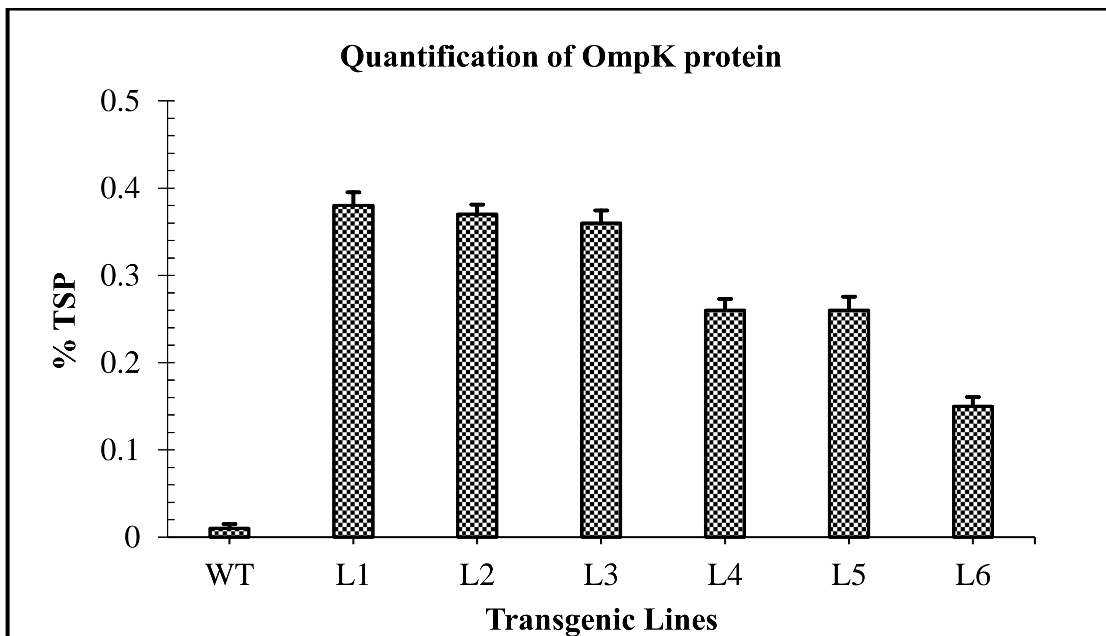



**Figure 4.16: OmpK protein quantification in transgenic plants.** TSP: total soluble fraction of leaf proteins; WT: wild type; L1-L6: six independent transgenic lines.

### 4.3.8. OmpK proves to be significantly immunogenic in mice and fish

To investigate the humoral response, the mice were subjected to immunization through both oral and subcutaneous routes. The antibodies against OmpK in the serum from immunized mice and fish were assayed by indirect ELISA. Promising results were shown by OmpK expressing transgenic tobacco plants when ELISA was conducted on collected blood sera. High IgG responses were shown in both mice groups F (oral delivery) and G (subcutaneous delivery). The antibody levels in the mice subcutaneously immunized were greater than those immunized via the oral route. The fish immunized orally with transgenic tobacco harboring OmpK also showed a significantly high level of IgG against its control groups (H&I). The IgG levels in fish were slightly lower than that of orally immunized mice group. Figure 4.17 shows the immunological study done on animal models.

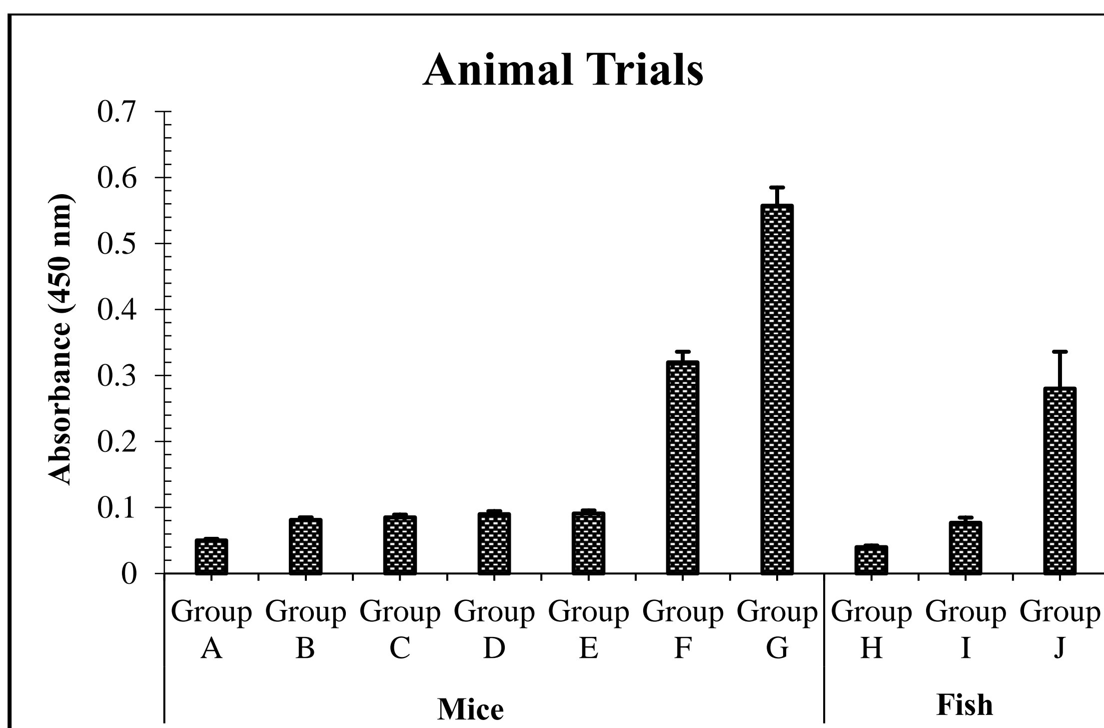



**Figure 4.17: Antibodies level in the blood sera of OmpK immunized animal models. Mice groups (A-G)** Group A: Normal mice (No dose); Group B: PBS solution oral delivery (control); Group C: PBS solution subcutaneous delivery; Group D: WT tobacco TSP oral delivery; Group E: WT tobacco TSP subcutaneous delivery; Group F: Transgenic tobacco TSP oral delivery; Group G: Transgenic tobacco TSP subcutaneous delivery. **Fish groups (H-J)** Group H: Normal group on commercial feed; Group I: WT tobacco oral delivery mixed in feed pellet; Group J: Transgenic tobacco oral delivery mixed in feed pellet.

**Annexure 4.1**

**Transformation media for tobacco leaves**

| Media | Ingredients | Concentration |
|---|---|---|
| **Co-cultivation** | Acetosyringone | 40 mg/L |
| | BAP | 1 mg/L |
| | Myo-inositol | 100 mg/L |
| | NAA | 0.1 mg/L |
| | Thiamine HCl | 1 mg/L |
| **Washing** | MS | 2.2 g/L |
| | Cefotaxime | 50 mg/L |
| **Selection** | BAP | 1 mg/L |
| | MS | 4.4 g/L |
| | NAA | 0.1 mg/L |
| | Sucrose | 30gm/L |
| | Thiamine HCl | 1 mg/L |
| | Myo-inositol | 100 mg/L |
| | Cefotaxime | 200 mg/L |
| | Hygromycin | 30 mg/L |

**Annexure 4.2**

**Protein extraction buffer for tobacco leaves for western blot**

| Components | Concentration |
|---|---|
| Sodium chloride | 100 mM |
| EDTA | 10 mM |
| Tris-HCl, pH 8 | 200 mM |
| SDS | 0.1% |
| β-mercaptoethanol | 15% |
| Sucrose | 200 mM |
| Polymethyl-sulfonyl-fluoride (PMSF) | 2 mM |

# *TRANSIENT EXPRESSION OF “OMPK” IN EDIBLE PLANTS*

*Chapter 5*

## 5.1. Introduction

### 5.1.1. Plant-based molecular pharming of edible vaccines

Plant-based edible vaccines, as defined by Lössl and Waheed (2011) and Naik (2022), are recombinant protein vaccines in which selected antigens are produced in certain plant species and administered orally in the form of an edible vaccine to elicit protective immunity against infections. Edible vaccines can be developed by the introduction of the gene encoding the protein, antigenic in nature, into the plant that would produce the immunogenic protein. This innovative approach enables the cost-effective distribution of vaccines globally via seeds. After edible vaccine ingestion, the antigenic protein traverses M cells, which are specialized cells to deliver antigens to dendritic cells and elicit immune responses (Concha *et al*., 2017; H. Mondal & Thomas, 2022).

### 5.1.2. Immunological mode of action of plant-based edible vaccines

The genetically engineered plant or plant part that is eaten orally and expresses the potential vaccine antigen passes through the mastication process. The majority of plant cell breakdown is brought on by the activity of the microbiome or by the action of digestive enzymes in the colon, which releases the vaccine antigens (Gunasekaran & Gothandam, 2020; H. Mondal & Thomas, 2022). Peyer's patches (PP) are a rich source of secretory immunoglobulin (IgA) which produces plasma cells, possessing the capacity to fill mucosal tissue and serve as an effector site for eliciting mucosal immunity (Buonaguro & Butler-Ransohoff, 2010; Streatfield, 2006; Takahashi *et al*., 2010). The disintegration of the edible vaccine close to PP, which is made up of lymphoid nodules on the gut's outer membrane and has follicles that grow the germinal center in response to antigenic stimulation. Antigen penetrates the intestine's epithelium through these follicles and gathers them in organized lymphoid tissues (Rybicki, 2010; Santi, 2009). The vaccine antigen is then in contact with the M- cells and any deep invaginations or pockets in the intestinal luminal cells' basolateral plasma membrane. B-cells, T-cells, and macrophages, among other immune system cells, are concentrated in these pockets. M-cells in these lymphoid follicles can express class II MHC molecules and deliver antigens across the mucosal membrane, which can activate B-cells (Daniell *et al*., 2009; Hefferon, 2010). When the activated B-cells reach the diffuse mucosal-associated lymphoid tissue (MALT), they go through plasma cell differentiation and release IgA subclass antibodies. These IgA antibodies enter the

secretions of the lumen through the epithelial cells and potentially bind to the antigen there (Dus Santos & Wigdorovitz, 2005; Yuki & Kiyono, 2003). The mechanism of action for the plant-based consumable vaccines is schematically depicted in Figure 5.1.

### 5.1.3. Advantages and disadvantages of plant-based edible vaccines

The edible plants' usage as a source of food and vaccine for both people and animals has multiple benefits. The manufacturing of vaccines using a plant-based system can give a biotechnological solution since it allows for high-yield, cost-effective production while also reducing issues with distribution and storage (Concha *et al*., 2017; Dadar *et al*., 2017; Embregts & Forlenza, 2016; Dhama *et al*., 2013). Plants can undergo post-transcriptional modification and proper folding of produced proteins, just like other eukaryotic species, and as a result, they can synthesize complex proteins. Although, they differ slightly from mammalian cells in terms of their glycosylation pattern (Kolotilin *et al*., 2014; Sohrab, 2020; H. Su *et al*., 2021).

The preservation of edible vaccines does not require a very cold environment. They can be supplied orally by directly consuming a specific transgenic plant part to protect against a particular infectious disease. In comparison to conventional vaccine production techniques like fermentation technology, plant-derived edible vaccines can be produced affordably in high bulk without requiring additional purification and processing stages (T. Das *et al*., 2021; Mičúchová *et al*., 2022). Transgenic seeds may be easily preserved for a long duration at room temperature and are also widely available as a source of vaccines. Thanks to the nuclear transformation technology at the plant, which has reportedly been responsible for producing vaccine antigens against several diseases caused by different pathogens, including viruses and bacteria that are dangerous to human health and animals (Cardi *et al*., 2010; Dadar *et al*., 2017).

There isn't a single plant-derived edible vaccination for animals that is commercially available, despite numerous publications showing the successful generation of vaccine antigens in plants (Waheed *et al*., 2016). Antigen expression levels in different areas of even the same plant vary, which is the fundamental constraint to figuring out the optimal dose. If the patient consumes too much plant-derived vaccine, it may result in hypertension and allergic responses. So, it is challenging to predict how much transgenic plant should be consumed to get the optimum dose of vaccine (Appaiahgari *et al*., 2017; Desai *et al*., 2010; Tiwari *et al*., 2009).

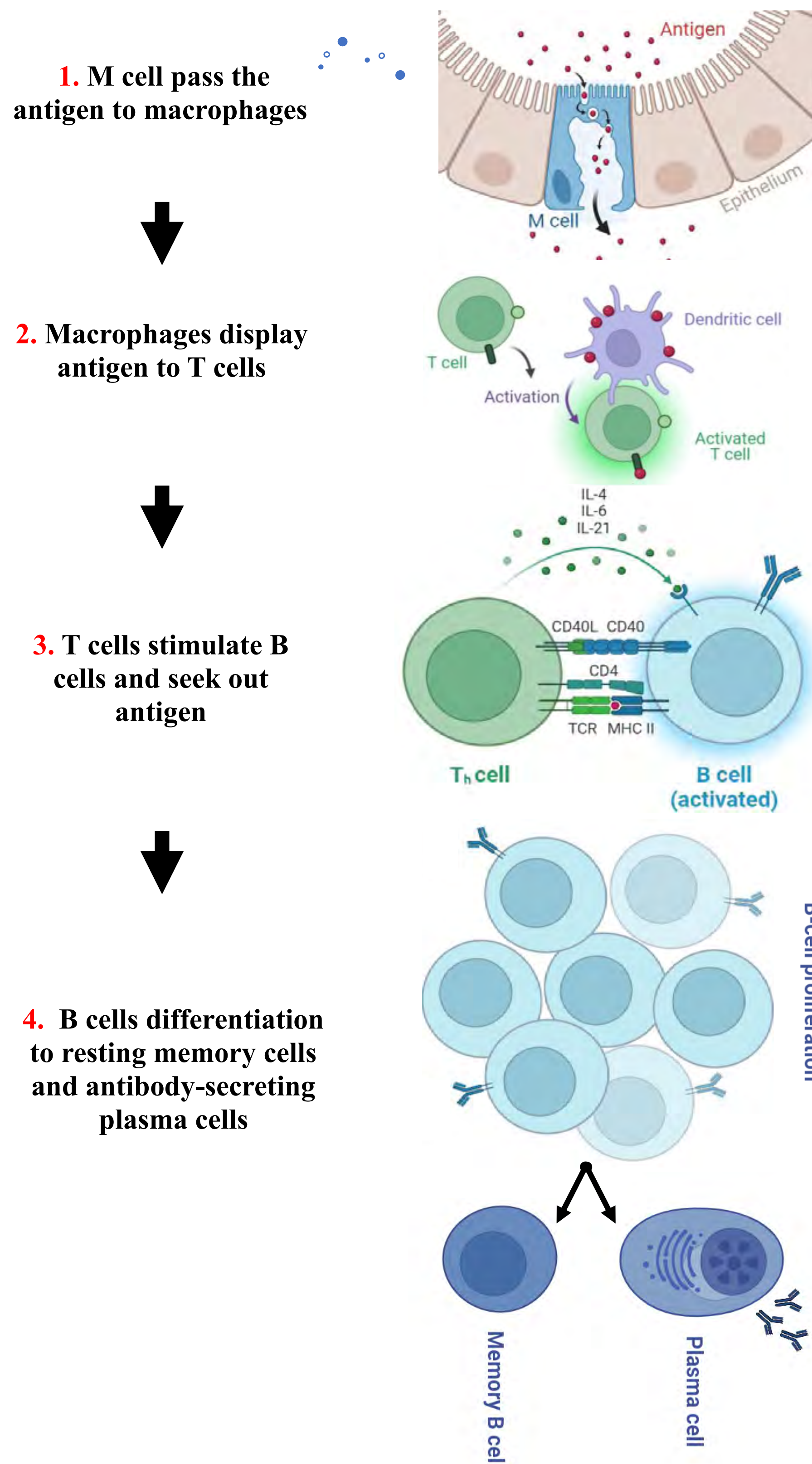



**Figure 5.1: The mucosal immunity path followed by plant-based edible vaccines** (Y. Gao & Guo, 2023; S. H. Kang *et al*., 2018; S.-H. Kim & Jang, 2014; Mbongue *et al*., 2023).

Major obstacles in the development of this technology are cross-pollination between transgenic plants and other crop plants, gene silencing issues, effects on soil microbes and insects, and disruption of the food chain (Tiwari *et al*., 2009).

### 5.1.4. Edible plants

Every human needs food to survive, and they either rely on plants directly or indirectly. Plants serve as the primary food and metabolic energy source for virtually all terrestrial and aquatic herbivorous animals, including humans, which are unable to produce their food. Edible plants are those that have portions that humans can comfortably eat. 20,000 species are known to be edible (Parodi *et al*., 2018). The most popular edible plants are cultivated across the globe in a variety of habitats and temperatures, both commercially and in backyard gardens. Leafy green vegetables (cabbage, spinach, and lettuce) and fruit crops (apples, bananas, oranges, and pineapple) are examples of small-scale crops, whereas crops farmed on a big scale often include maize, rice, wheat, oats, and potatoes (Blancke, 2016). Different vegetable varieties are grown specifically for their leafy sections including, cabbage, mustard greens, spinach, Swiss chard, and turnip greens. Leafy vegetables' leaves can be consumed as raw, sometimes along with petioles and shoots. There are more than a thousand edible species of short-lived herbaceous plants, including spinach and lettuce (French, 2015).

### 5.1.5. Plants as fish feed

The demand for quality fish food is continuously increasing as the aquaculture growth is rapidly increasing to fulfil the fish food demand. The main aim of the fish farmers is to provide fish the high-quality feed, but this costs them around 50% of total production expenses. Despite this huge cost quality feed plays a pivotal role in overall yield and profit (Dorothy *et al*., 2018; Mzengereza *et al*., 2014). The quest for potential alternative protein sources, aimed at partially or completely replacing commercial animal-based protein fish feed, became paramount. This is due to its limited availability in the market and inflated costs. Therefore, plants serve as a potential alternative food source for fish without compromising the nutritional quality of the feed. Furthermore, locally affordable plants as a substitute for expensive fish meals would lead to a reduction in cost, thereby enhancing overall profitability (El-Sayed, 1999; Francis *et al*., 2016; Magouz *et al*., 2008; Munguti *et al*., 2006; Osman *et al*., 1996). Plants which are rich in proteins with diverse amino acids and fatty acids which lack animal protein

have been explored as fish species-specific feed for various commercial fish species cultured in aquaculture based on their requirement. This strategy offers advantages in availability and cost-effectiveness. Additionally, plant-based feed has lower levels of phosphates and nitrogen than animal-based feed and thus has reduced pond eutrophication risks (Dorothy *et al*., 2018; K. Mondal & Payra, 2015).

The key factors during the choice of plants as fish feed are acceptability, digestibility, and palatability by fish. Plant-based fish feed's main limitation is its low protein content, and anti-nutritional elements' presence (alkaloids, cyanoglycosides, glycosides, haematoglutinin, momosine, oxalic acids, phytates, protease inhibitors), and imbalances in essential amino acids, fatty acids, and micronutrients. Despite these disadvantages, the benefits due to plant feed cannot be ignored (Abowei & Ekubo, 2011; Dorothy *et al*., 2018). The effect of anti-nutritional elements can be mitigated by leaf meals by soaking them in water, followed by drying and grinding in small particles (Anderson & Wolf, 1995; Bairagi *et al*., 2002; Lochmann *et al*., 2011; Mzengereza *et al*., 2014). Plant leaves, stems, seeds and tubers and their extracts can be used as fish feed. Terrestrial plant leaves can often be used as fish feed (Bardach *et al*., 1974; K. Mondal & Payra, 2015). Numerous studies have been done on the use of different parts (leaves, stems, seeds, or tubers) or extracts of parts or whole plant of akee, aquatic weed, blackjack, banana, broad beans, cassava, cereals and cereals by-products, cocoyam, corn gluten, cottonseed, cowpea, cucumber, duckweed, fruits of certain plants, grasses, jackfruit, maize, Mexican fire plant, moringa, mung-bean, olive cake like linseed, papaya, peanut, rapeseed, rice (broken and polished), safflower, sarliconia, soybean, sorghum, sunflower, sweet potato, wheat, etc. as fish feed (Dorothy *et al*., 2018; Egwui *et al*., 2013; Francis *et al*., 2016; Magouz *et al*., 2008; K. Mondal & Payra, 2015; Mzengereza *et al*., 2014; Vhanalakar, 2009).

### 5.1.6. Selection of Spinach and lettuce for transient expression

Plants can be used as cost-effective fish feed and as their mass production is cheaper (Abdel-Hakim *et al*., 2008; Santigosa *et al*., 2008). Therefore, researchers considered them essential for continuous aquaculture and for this reason, several studies have been carried out to include them in fish feed (Gatlin III *et al*., 2007; Glencross *et al*., 2007). Spinach and lettuce are leafy vegetables that can be used as edible fish vaccines. The taxonomic classification of both *Spinacia oleracea* L. and *Lactuca sativa* L. is given in Figure 5.2.

Spinach (*Spinacia oleracea* L.) is the world's healthiest vegetable, according to WHO. It is rich in vitamins and minerals and has beta-carotene, lutein, xanthin, and flavonoids offering antioxidant protection. Spinach is distinctive among green vegetables due to its high nutritional value, antioxidant qualities, and vitamin content (Swiader *et al*., 1992; Zdravković-Korać *et al*., 2023). One study was conducted to assess how β-carotene from two natural sources viz., carrot (*Daucus carota*) and spinach affect the coloration of *Xiphophorus helleri*. The finding revealed that the fish fed with spinach exhibited more yellow-colored pigments, while those fed with carrot displayed enhanced red-colored pigmentation, suggesting that feed incorporated with a natural β-carotene source can be employed as a cost-effective color enhancer (Wagde *et al*., 2018). Similarly, the study carried out by Patel *et al*. (2023) also confirms the spinach (powder form) as an excellent growth and colour enhancer in the *Trichopodus trichopterus*. Another study suggests the use of *S. oleracea* as an antibiotic instead of commercial antibiotics to fish farmers to obtain healthy fish production (Sridhar & Esther Joice, 2018). Ayoub and Khames (2019) concluded that the use of a mixture of vitamin E and spinach in the diet of *Oreochromis niloticus* can enhance growth and elicit non-specific immunogenic responses.

Lettuce (*Lactuca sativa* L.), a member of family *Asteraceae* family, is globally cultivated and primarily consumed in its raw due to its appealing taste, affordability, and high nutritive value, especially for its fiber, mineral input, and vitamin in a diet (Ahmed *et al*., 2021; Mulabagal *et al*., 2010; Sapkota *et al*., 2019; Xylia *et al*., 2021). Several studies have been carried out to explore the potential of *L. sativa* as fish food (Z. Fischer, 1972, 1973; Mandal *et al*., 2010; Van Dyke & Sutton, 1977). Studies focused on evaluating the efficiency of fish to absorb lettuce revealed that *Ctenopharyngodon idella* exhibited a 20% absorption efficiency when exclusively fed with the lettuce plant (Z. Fischer, 1972, 1973; Mathavan *et al*., 1976). Another study incorporating 8-12% dried lettuce leaf powder in fish feed to replace wheat and oats resulted in improved growth indicators and survival rates without negative effects in white fish *Rutilus kutum* (Rafiee *et al*., 2018). This work can be beneficial to produce fish food at a lower cost. In another study treatment of Piranha fish (*Pygocentrus nattereri*) with lettuce (100%) showed 168% of weight gain at a 3.57 growth rate percentage with a 97% survival rate (Mirzabagheri *et al*., 2016). These above-

mentioned studies show that lettuce and spinach can be the potential plant-based platform for edible vaccine development.

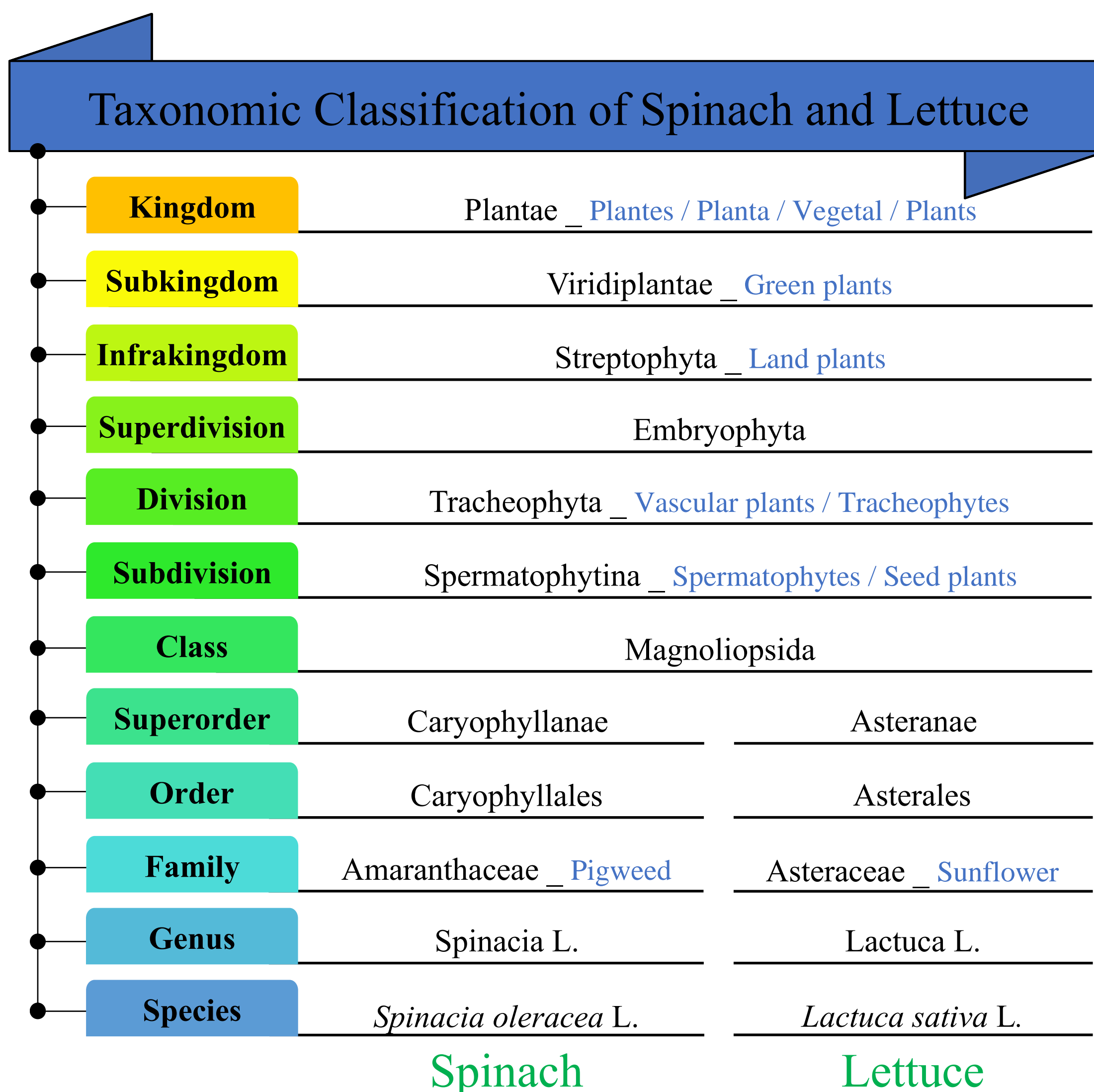



**Figure 5.2: Scientific classification of *Spinacia oleracea* L. and *Lactuca sativa* L.**

### 5.1.7. Spinach

Spinach is a green economically annual, wind-pollinated, dioecious plant being a member of the family Amaranthaceae. It is grown around the world for its edible leaves. The "spinach" name originated from "ispanai"(a Persian word) which means "green hand" (Madhvi *et al.*, 2014; Ramaiyan *et al.*, 2020). Spinach is a temperate area plant and grows at 15-18°C in the spring or the fall with the maximum leaf production at these conditions. The germination temperature for seeds is 4°C. Spinach is a plant that grows under lengthy days with a critical day length of 13–14 hours that's why four to eight photoperiods are necessary for induction (Chitwood, 2016). The minerals found

in spinach leaves, which make up to 1.8 per cent of the dry weight, are present in all leafy vegetables and are crucial sources of nutrition. Due to the relatively high iron content of spinach leaves (4-6 mg per 100g dry weight), which gives the vegetable its unique nutritional value, spinach is advised for people with anemia and young children (Murcia *et al*., 2020; Rashid *et al*., 2020). Other agricultural important crops in this family of the Caryophyllales order include beet, quinoa, and amaranth (Hassler, 2019).

Although its exact origins are unknown, it is thought that domesticated spinach was first developed in the region of Iran, former Persia, some 2,000 years ago. Since no references to spinach have been discovered in Greek or Roman writings, and since the earliest known written accounts of the crop date from the fourth century A.D. in Mesopotamia, it is assumed that the crop has only recently become widespread. Spinach likely entered China via Nepal according to records dating back to the 7$^{th}$ century. Although accounts claim that the Saracens brought spinach to Sicily in the 9$^{th}$ century, the first recorded accounts of its cultivation in Muslim lands originate from the 10$^{th}$ century, and the first documented accounts of its cultivation in continental Europe date from Moorish Spain in the 12$^{th}$ century (El Faïz, 1995; Frye, 1962; Hallavant & Ruas, 2014; Heine, 2018; Ribera *et al*., 2020; Rolland & Sherman, 2006).

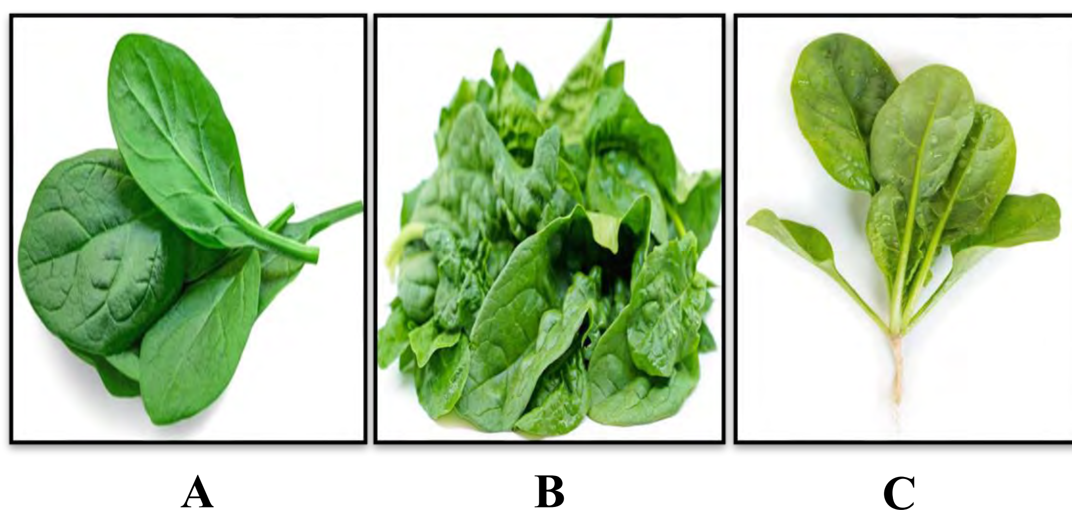
Two major cultivars of spinach Western-type and Asian-type cultivars are often distinguished from one another. These differences are likely due to the various selection regimes used in the two regions. Recent phylogenetic analyses have proved a relationship between spinach accessions' genetic relationship and geographical origin, with recurrent distinctions between these Western and Asian cultivars. Asian cultivars maintain the wild spinach's long petioles, and smooth, hastate, and narrow leaf shape, while Western cultivars have round and enlarged leaf texture (Savoy leaf) (Ribera *et al*., 2020, 2021; She *et al*., 2018; C. Xu *et al*., 2017).

#### 5.1.7.1. Morphology and types of spinach

Annual plants like spinach have distinct vegetative development and reproductive stages. Usually, seeds are sown during the last days of winter or in the early days of spring. The rosette-shaped leaves can have crinkly or flat appearances (J. Ma *et al*., 2016). Simple leaves of the spinach plant have an approximate length and width of 2–30 cm (0.8–12.0 in) and 1–15 cm (0.4–6.0 in), respectively. These leaves grow from the center of the plant. The shrub bears 3-4 mm (0.1 in) wide, yellow to green, little

blooms (Ebadi-Segheloo *et al*., 2014). The flowers grow little fruit clusters with seeds in them. Spinach plant that lives for just one growing season can attain a maximum height of 30 cm. Originally from Iran, spinach is a product of ancient Persia (Eftekhari *et al*., 2010; Meng *et al*., 2017).

Spinach, based on morphology, has three main types. The most popular type of spinach is flat-leaf spinach also known as smooth leaf spinach or baby spinach. It has spacious, spade-shaped leaves, a delicate texture, and a somewhat sweet flavor. The second type is Savoy spinach. It has curly, crinkly, rigid, and dark green leaves. The third kind is Semi-savoy spinach which is a hybrid variety of savoy spinach and flat-leaf spinach. It has somewhat crinkly leaves but is not crispy (Grevsen & Kaack, 1997). The two main forms of spinach are the smooth leaf variety and the crinkle leaf variety, and they range in color from light to dark green (Eftekhari *et al*., 2010; Meng *et al*., 2017). Figure 5.3 depicts all three types.



A B C

**Figure 5.3: Distinct types of *Spinacia oleracea* L. (A)** Flat-leaf spinach **(B)** Savoy spinach **(C)** Semi-savoy spinach.

#### 5.1.7.2. Medicinal uses of spinach

Since ancient times, *S. oleracea* (green leafy vegetable) has been used as a food source and includes biological elements that have significant pharmacological or therapeutic value. These biological elements are beneficial for preserving human health including defense against eye problems (as it has vitamin A), oxidative stress, iron deficiencies, etc. and lower the chance of developing certain diseases like diabetes, cancer, and hepatotoxicity (Jiraungkoorskul, 2016; Mane *et al*., 2015; Nayak *et al*., 2010; Tewani

*et al*., 2016). Spinach has vitamin E and a high content of magnesium which helps strengthen the immune system against viruses and bacteria and protects from contaminants and toxins (Mukherjee *et al*., 2016).

Spinach has vitamin C, therefore helps to build collagen, which is necessary for healing wounds. In addition to supporting the healing process, vitamin C also increases the amount of iron that the body absorbs from plant-based diets (Güler *et al*., 2015). High quantities of nitrates found in spinach have been demonstrated to help maintain blood pressure and reduce the risk of cardiac disease (A. H. Liu *et al*., 2013). Vitamin K is an important constituent of spinach and helps in keeping strong bones. Spinach also helps the body absorb calcium and helps in bone and teeth strengthening (Adhikary *et al*., 2017). Neoxanthin and violaxanthin, two anti-inflammatory components that control inflammation, are present in this superfood (Garg *et al*., 2010).

#### 5.1.8. Lettuce

*Lactuca sativa* L. is a green leafy edible crop, mainly consumed as fresh salad, cultivated around the world. In addition, some forms are also cooked (Křístková *et al*., 2008; H. Peng & Simko, 2023; Said, 2012). Lettuce is a member of the *Asteraceae* family and falls under the genus *Lactuca*. The genus *Lactuca* is known to encompass approximately three hundred species (Madina & Akinyemi, 2023; Mohebodini *et al*., 2011; Ryder, 2012). Lettuce is popular as a low-calorie food consisting of 95% water and various nutrients (iron, copper, sodium, and phosphorus). The nutritional value of lettuce is mainly based on the morphological type, colour, and orientation of leaves. Lettuce is hierarchical at seven positions as compared with other vegetables and fruits in terms of nutrient-rich contents (Di Noia, 2014; Mohamed *et al*., 2021; Sammour, 2014; Shi *et al*., 2022; X. Yang *et al*., 2022). Subject to the species, lettuce is a rich source of vitamins (A, C and K), and Calcium in dark green cultivars along with copper, and iron (R. Das & Bhattacharjee, 2020; D. Kumar *et al*., 2020; Rafiee *et al*., 2018). According to the U.S. Department of Agriculture (USDA) (2022) depending upon the variety of lettuce, the level of dietary fibers, carbohydrates, proteins, and fats varies.

##### 5.1.8.1. Morphology and types of lettuce

Lettuce is an annual or biennial lactiferous self-pollinating herb. It ranges in height from 6-12 inches or 15-30 cm. It has a diverse and dense root system. These roots grow more in a horizontal direction rather than a vertical direction except for taproot (Hassan *et*

*al*., 2021; Křístková *et al*., 2008). The lettuce plant has a short thick stem of about 10-50 cm depending on the type of lettuce. The stem is branched at the apex. Lettuce plants have hairless leaves that may be smooth, crumpled (wrinkled) or savoy (wrinkled and curled). These leaves vary in colour ranging from a very dark green to a very light green color depending on the type. There are few varieties of lettuce having yellow, red, gold, or blue-teal leaves (DuPont *et al*., 2000; Han *et al*., 2021; Hassan *et al*., 2021; Křístková *et al*., 2008). Lettuce to endure flowering grows a single stem. Flower head (capitula), bears multiple florets (12-20). Each floret has a different calyx (pappus), a corolla (five petals fused to a ligule) and the reproductive parts (bipartite stigma and anthers fused in tube form around a style). The ovaries make compressed and obovate dry fruits having a length of 3-4 mm. Each side of the fruit bears 5-7 ribs and two rows of tiny white hairs at the tip. The pappus acts as a dispersal structure and remains at the apex of the fruit. A single seed is present in each fruit which may be yellow, white, brown, or grey depending upon the type of lettuce (De Vries, 1997; Křístková *et al*., 2008).

Lettuce is characterized into the following types based on their growth and shape, consisting of (i) Butterhead, (ii) Crisphead (Iceberg), (iii) Latin, (iv) Loose leaf (Cutting), (v) Oilseed, (vi) Stalk (Asparagus), and (vii) Romaine (Cos) (Mampholo *et al*., 2016). The frequently cultivated types of lettuce are shown in Figure 5.4. The taxa of *Lactuca* is mostly dispersed in the Northern Hemisphere, Mexico to North America, New Guinea to Asia, and Africa to South Africa (Hassan *et al*., 2021; Kadereit & Jeffrey, 2007; Wani *et al*., 2020).

#### 5.1.8.2. Medicinal uses of lettuce

Several medical uses of lettuce are reported in literature and some of them are mentioned in this section. Due to the presence of a great concentration of dietary fibre in lettuce, it is usually advised for patients with constipation. Lettuce is also recommended for patients who suffer from mild-to-moderate forms of anxiety and sleeping difficulties, as it contains a sleep-inducing substance. In ancient times its seed oil has been used in folk medicine as a sleeping aid and to relieve pain and inflammation (Shi *et al*., 2022; Yakoot *et al*., 2011). Lettuce can be used to heal medical problems such as Alzheimer's disease, cancer, diabetes, and oxidative damage. The lettuce's anti-diabetic properties may be due to its very low carbohydrate content and high magnesium content. As high magnesium content reduces the risk of type-2 diabetes

(Bahbah *et al*., 2021; Kabir *et al*., 2021; Larsson & Wolk, 2007; Naseem & Ismail, 2022).

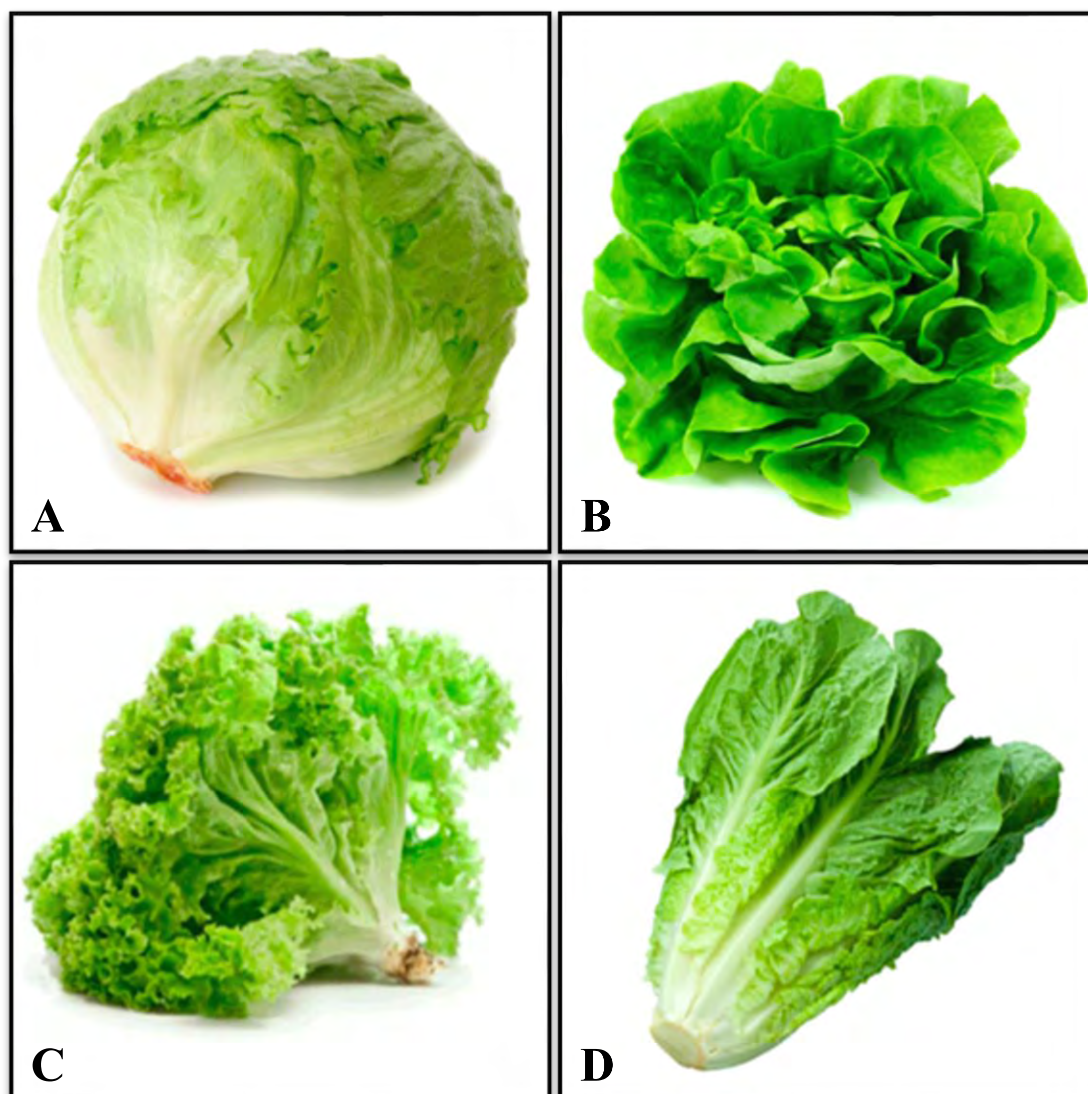



**Figure 5.4: Distinct types of *Lactuca sativa* L. (A)** Crisphead **(B)** Butterhead **(C)** Loose-leaf **(D)** Romaine.

Lettuce leaves have high carotenoid content (β-carotene, lactucaxanthin, and lutein), while lactucaxanthin is not common in other plants and it inhibits α-amylase and α-glucosidase activities making lettuce anti-diabetic (Gopal *et al*., 2017; Shi *et al*., 2022). *L. sativa* anti-cancer properties can be due to rich in antioxidants compounds such as ascorbic acid, caffeic acid, carotenoids (β-carotene), chlorogenic acid, deoxylactucin, dicaffeoyl tartaric acid, guaianolide sesquiterpene lactones, lactucin, lactucopicrin, phytols, polyphenols, quercetin, and vitamin C (Materska *et al*., 2019; Shi *et al*., 2022; Sularz *et al*., 2021; Tareq *et al*., 2020). Lettuce may function as an immunomodulator

due to polysaccharides (SLP-1 and SLP-2). SPL-1 has galacturonic acid, galactose, and arabinose in abundance while SLP-2 has mannose, galacturonic acid, galactose, and arabinose as primary constituents. Both also have sulfate radicals. Because of SLP-1 and SLP-2 which enhances the ability of lettuce to proliferate macrophages (Hefnawy & Ramadan, 2013; Nie *et al.*, 2018; Seo & Jeong, 2020). The hepatoprotective effect of ethanolic extract of *L. sativa* has been reported during $CCl_4$-induced toxicity in rats and researchers concluded that this is due to the presence of free-radical scavenging antioxidants in lettuce. Due to isochlorogenic acid (phenolic compound) and glycoside B, lettuce also possesses neuroprotective activity (Im *et al.*, 2010; Zhan *et al.*, 2014). Regular intake of lettuce helps in blood clotting, reduces bone loss, and heals bruises because of vitamin K. There are also reports of anti-ageing behavior of lettuce in case of the nervous system due to polyphenols and in case of the skin due to Vitamin A and C (S. Chaudhary, 2015; Rizvi & Maurya, 2007; Shi *et al.*, 2022). For other digestive disorders like gastric pain, it is recommended to take lettuce before meals as it helps in digestion due to the presence triterpene lactones (an anti-inflammatory agent) (Araruna & Carlos, 2010; Sayyah *et al.*, 2004).

### 5.1.9. Transient transformation of plants

Plant cells can be genetically modified to express genes either temporarily or permanently. In plants, transient expression serves as a valuable tool across various biochemical manipulations, functional genomics, promoter testing, and protein subcellular localization applications. It can be used for both the upregulation and downregulation of understudy genes. Transient gene expression usually lasts for a noticeably brief time. It is also a scalable and feasible alternative to animal cell culture and microbial fermenter-based systems for recombinant protein production. New and modified proteins and pharmaceutical substances including human therapeutic proteins, vaccines, and antibodies can be transiently expressed in plants (Jones *et al.*, 2009; Newell, 2000; Shen *et al.*, 2014). Recombinant DNA's transient introduction into cells of plants can be achieved via *Agrobacterium,* physical methods, or viral vectors. *Agrobacterium tumefaciens*, which initiated plant transformation has an inherent ability to modify host plants (Figure 5.5). *Agrobacterium*-mediated transient transformation is a proposed method for transient expression (Chilton *et al.*, 1977; Janssen & Gardner, 1990, 1993; Jones *et al.*, 2009; Krenek *et al.*, 2015). The main steps in *Agrobacterium*-mediated transformation are: (1) signal from plant cell recognized by *Agrobacterium*

*tumefaciens*, (2) processing of T-DNA, (3) migration of T-DNA into the host cell, (4) integration of T-DNA into the plant nuclear genome, and (5) expression of inserted T-DNA in plant host cells (Guo *et al*., 2019; Iwakawa *et al*., 2017; Krenek *et al*., 2015; Lacroix & Citovsky, 2013, 2019, 2020; Nishizawa-Yokoi *et al*., 2021; Ozyigit, 2020; Özyiğit, 2012; Pitzschke & Hirt, 2010; Saika *et al*., 2014).

In case of transient transformation through *Agrobacterium*, transgene transfer and expression occur without the integration of Ti-plasmid. It does not rely on heterologous DNA chromosomal integration. Therefore, transient transformation is a facile method and therefore high level of transgene expression can be achieved (Gelvin, 2010; Jones *et al*., 2009). When *A. tumefaciens* interacts with plant cells or tissues, a single-stranded copy of T-DNA is complexed with proteins and transferred into the nucleus of the plant tissues. The inserted T-DNA copies are only momentarily present in the cell nuclei and do not integrate into the plant genome. The nuclear transcription of the non-integrated T-DNA copies results in a temporary expression for a few days which typically peaks between 2-4 days after infection and lasts for 10 days, declining gradually for each transformed plant cell. (Joh *et al*., 2005; Krenek *et al*., 2015; Sainsbury, 2020; Sainsbury & Lomonossoff, 2014). A high level of transient expression in infected plant cells or tissues may be observed because initially plant cells acquired T-DNA with large copy numbers from *Agrobacterium*, resulting in maximal transient expression which then gradually diminished due to instability of injected non-integrated T-DNA copies (Lacroix & Citovsky, 2013; Sánchez-Álvarez *et al*., 2019).

#### 5.1.9.1. Advantages and disadvantages of transient expression

Transient expression systems have multiple benefits, including cost-effectiveness and speedy and high transgene expression in a limited amount of time. The only place where cells are damaged with this non-invasive process is where the infiltration occurs. This technique is highly flexible because different parts of the sample can be infiltrated with one or more constructs, allowing the same infiltrated sample to be used for multiple experiments (Heenatigala *et al*., 2018; Nosaki & Miura, 2021). The technique has a few drawbacks, including transgene loss due to host cell replication and the need for re-infection because the expression is transient (Y. Li *et al*., 2021; Y. Zhang *et al*., 2020).

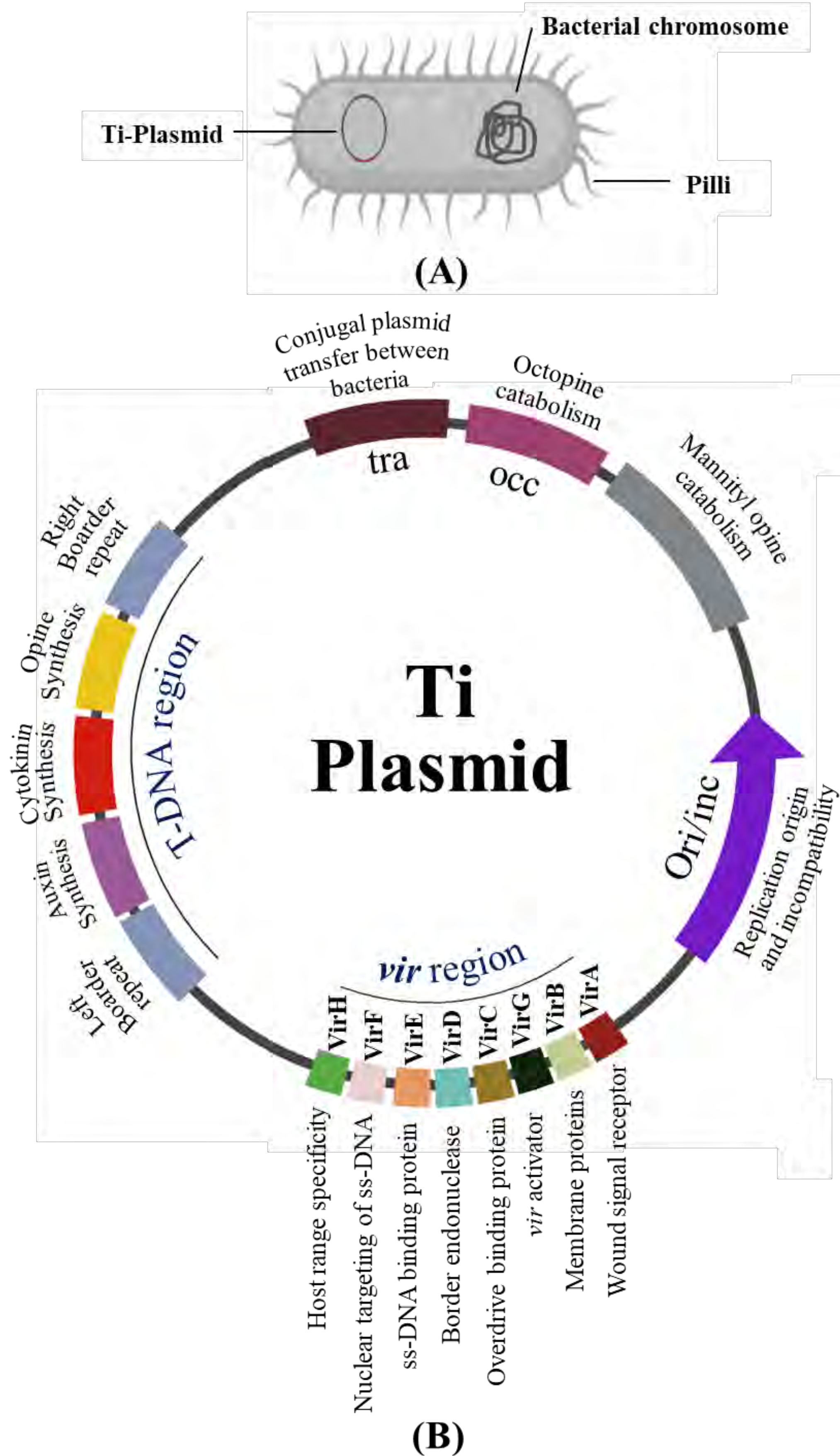



**Figure 5.5: (A)** *Agrobacterium tumefaciens* **(B)** Ti-plasmid. (Özyiğit, 2012)

### 5.1.10. Objectives

In the present part of the work, the aim was to transiently express the OmpK antigen into edible plants (Spinach and lettuce) for the possible development of a plant-based edible vaccine against vibriosis through *Agrobacterium*-mediated transient transformation.

## 5.2. Materials and methods

### 5.2.1. Plant growth

Seeds of *S. oleracea* L. (F1 hybrid) and *L. sativa* L. (Grand Rapid) were obtained from Awan seed store, a well-known store found in territory of Islamabad, Pakistan. Seeds were stored at a cool and dry place with relative humidity (< 30%) and a temperature of 20°C. The seeds of both edible plants were rinsed with double distilled autoclave water thrice. Then sowed in autoclaved mud supplied with natural fertilizers under optimum conditions of light (16 hours) and dark (8 hours) in a growth room at 25±1°C.

### 5.2.2. *Agrobacterium* infiltration media preparation

OmpK-binary vector transformed *A. tumefaciens* (GV3101) was spread on YEB (Annexure 5.1) agar plates containing kanamycin and tetracycline and incubated for 48 hours at 25±1°C. then 5 mL of YEB culture containing kanamycin and tetracycline was inoculated with transformed *A. tumefaciens* (GV3101) and was allowed to grow at 28±1°C overnight at 200 rpm in a shaking incubator. 1.5 mL of culture was transferred to a sterile 2 mL tube and subjected to centrifugation at 1,000 x g for 10 minutes at 24±1°C to obtain a bacterial pellet by removing the supernatant. The bacterial pellet was resuspended in 1 mL of infiltration media (Annexure 5.2). The centrifugation process was repeated three times to remove any left antibiotic in infiltration media to prevent plant leaves from being damaged due to antibiotics. The absorbance of a 1 in 10 dilution of cells was evaluated at 600 nm, and the initial concentration OD was calculated by multiplying it by ten. The cultures whose $OD_{600nm}$ value was more than 1.5 were used to get maximum transient efficiency. The titer of culture was determined for infiltration by the below-mentioned formula given by Sparkes *et al.* (2006) with the final concentration OD to be 1.0.

$$\textbf{Initial volume}\ (\mu \boldsymbol{L}) = \frac{(\textbf{Final concentration}\ (\boldsymbol{OD}) \times \textbf{Final volume}\ (\mu \boldsymbol{L})}{\textbf{Initial concentration}\ (\boldsymbol{OD})}$$

The resulting infiltration media was used for infiltration.

### 5.2.3. *Agrobacterium* infiltration of plants

Both plants (Spinach and lettuce) pots from the growth chamber were kept for 1 hour under a white-fluorescent lamp before infiltration to fully open stomata to obtain efficient transient transformation. Healthy large leaves (3-4 weeks old) were chosen for infiltration as it is harder to infiltrate small leaves. The leaves were labelled with a marker for easy identification of infiltrated leaves and leaves were gently rubbed to make them ready for infiltration. The leaves were infiltrated in two midrib regions. The infiltration media was taken in the 1 mL syringe without a needle. Then gently infiltration media was pressed down in the leaves from the dorsal side until the infiltration media fully diffused into the whole leaf. The infiltrated area was then again marked to select only the infiltrated area in molecular analysis. After successful infiltration, the plants were placed in the growth chamber under optimum conditions.

### 5.2.4. Collection of leaves

Different co-cultivation time was provided for leaves. Leaves were picked on the 1$^{st}$, 2$^{nd}$, 3$^{rd}$, 4$^{th}$, and 5$^{th}$ days post infiltration. They were then kept at -20°C while being wrapped in aluminum foil.

### 5.2.5. Molecular analyses

#### 5.2.5.1. Confirmation of infection by PCR

To confirm infection in the leaves of both edible plants PCR was performed. The method for isolation of bacterial plasmid is described in detail in Chapter 2 section 2.2.6.1. The genomic DNA from the infiltrated and non-infiltrated leaves of both edible plants (spinach and lettuce) was extracted via hexadecyltrimethylammonium bromide (CTAB) proposed by Murray and Thompson (Murray & Thompson, 1980). The details protocol has been described in Chapter 2 section 2.2.6.2. PCR was performed by using ThermoFisher Scientific (USA) Taq polymerase standard PCR Kit. 1 µM of both primers (OmpK-IF primer: 5'_CTAAGCAACCCAAGCAGTGACAAAG-3' and OmpK-IR primer: 5' CCTTCATCTTTCAGACCATAAACATCTTTGTAGC-3'), and 100 ng of template DNA was used for confirmation of infection within infiltrated and non-infiltrated leaves. The PCR conditions, annealing temperatures and expected fragment size are given in Tables 2.5, 2.9, and 2.10 of Chapter 2. The bands were visualized using a gel documentation system (FluorChem FC3, USA).

#### 5.2.5.2. Protein extraction from infiltrated leaves

Leaf material from infiltrated and non-infiltrated leaves (100 mg) was grounded in liquid nitrogen and then re-suspended in 400 µL of protein extraction buffer (Annexure 3.2 of Chapter 3) to extract total soluble protein (TSP). The homogenized sample was centrifuged at 20 × 1000 g at 4°C for 10 minutes. The supernatant was collected and was regarded as a soluble fraction of protein. Total protein concentration in the soluble fraction was figured out via Bradford assay against bovine serum albumin (BSA) as standard (discussed in detail in Chapter 2).

#### 5.2.5.3. Western blot analysis

The protocol of Western blotting has been described section 2.2.11 of Chapter 2.

#### 5.5.5.4. Quantification of transgenic protein via ELISA

Protein extracts were prepared in extraction buffer (Annexure 2.9 of Chapter 2) from the fresh leaf tissue of the infiltrated. The homogenized sample was centrifuged at 20,000 g at 4°C for 10 minutes and the supernatant (SF) was collected. This supernatant was further used for the transgenic protein (TP) quantification via ELISA by following the protocol given by given in detail in section 2.2.13 of Chapter 2. The amount of TP as a percentage of the TSP of transformed leaf material was calculated by the formula:

$$\% \boldsymbol{TSP} = \left(\frac{\boldsymbol{TP}}{\boldsymbol{TSP}}\right) \times \mathbf{100.}$$

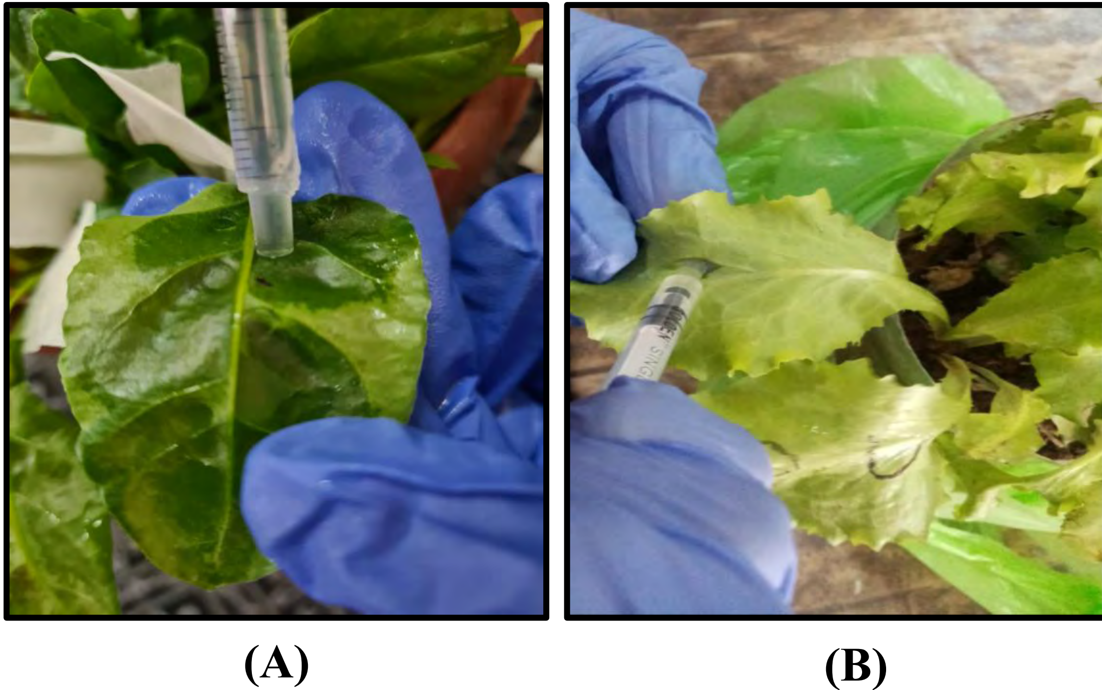
## 5.3. Results

### 5.3.1. Seed germination

The experiments of the current study were conducted by using seeds of *Spinacia oleracea* L. and *Lactuca sativa* L. After sterilization seeds were sowed in the pots. The mud used was autoclaved and then supplemented with natural fertilizer.

### 5.3.2. *Agro*-infiltration of leaves

Leaves of *S. oleracea* L. and *L. sativa* L. were infiltrated with *Agrobacterium* having binary vector pGWB5 having OmpK. Leaves were infiltrated by protocol mentioned by Sparkes *et al*. (2006). They were picked on different days i.e., on the 1$^{st}$, 2$^{nd}$, 3$^{rd}$, 4$^{th}$, and 5$^{th}$ day of *Agro*-infiltration and were used for PCR confirmation and gene expression analysis (Figure 5.6).



(A) (B)

**Figure 5.6:** ***Agro*-infiltration (A)** Spinach F1 hybrid **(B)** Lettuce Grand Rapid.

### 5.3.3. OmpK protein confirmation via PCR

DNA was extracted from the leaves by following the protocol given by Murray and Thompson (1980), with few modifications. The presence of transgene (OmpK) was confirmed by conventional PCR. Specific primers were used for confirmation of transgene in our leaf samples. Figures 5.7 (A) and (B) show a gel image with 500 bp bands of amplified PCR product of non-infiltrated vs infiltrated samples. The expected bands were seen on all five days of leaf samples. This confirmed the presence of T-DNA copies in the infiltrated leaves. Figure 5.7 (A) shows that the T-DNA expression diminished on the $5^{th}$ day in the case of spinach.

### 5.3.4. OmpK protein confirmation via western blotting

Western blotting was used to confirm the expression of OmpK protein in infiltrated leaves. The monomeric form of OmpK with a size equal to 30.21 kDa was detected in 1-4 days samples in the case of spinach, while in the case of lettuce expected band on OmpK in all five days samples was detected confirming successful transient transformation in both edible plants. No bands were seen for the non-infiltrated sample. Figure 5.8 shows the western blot-based confirmation of OmpK protein in both plants (*S. oleracea* L. F1 hybrid and *L. sativa* L. Grand Rapid).

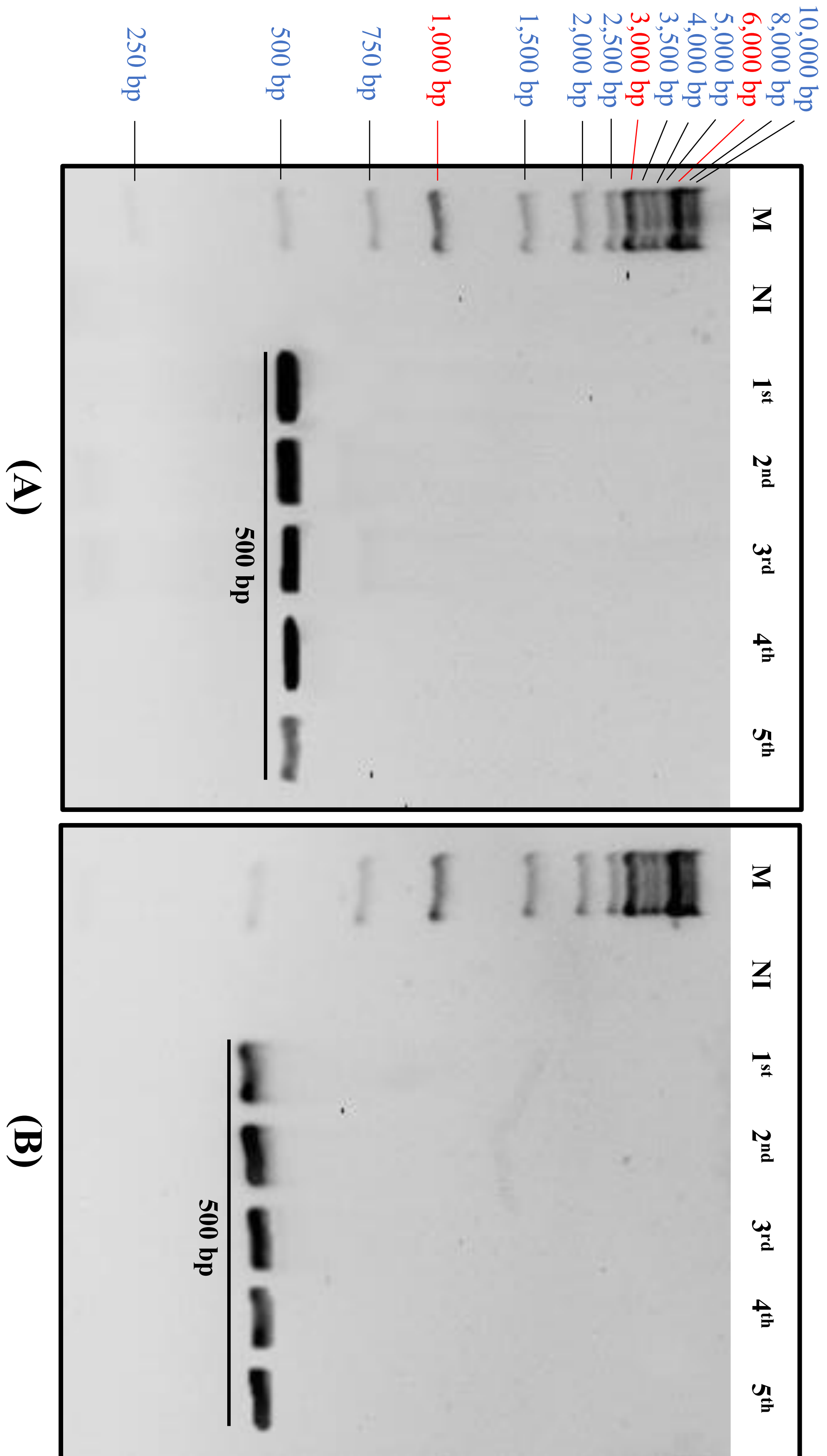



**Figure 5.7: Confirmation of transgene in infiltrated leaves. (A)** Leaves samples of *Spinacia oleracea* L. (F1 hybrid) **(B)** Leaves samples *Lactuca sativa* L. (Grand Rapid). M: 1 kb DNA marker; NI: Non-infiltrated leaves; 1-5: Infiltrated leaves collected on different days.

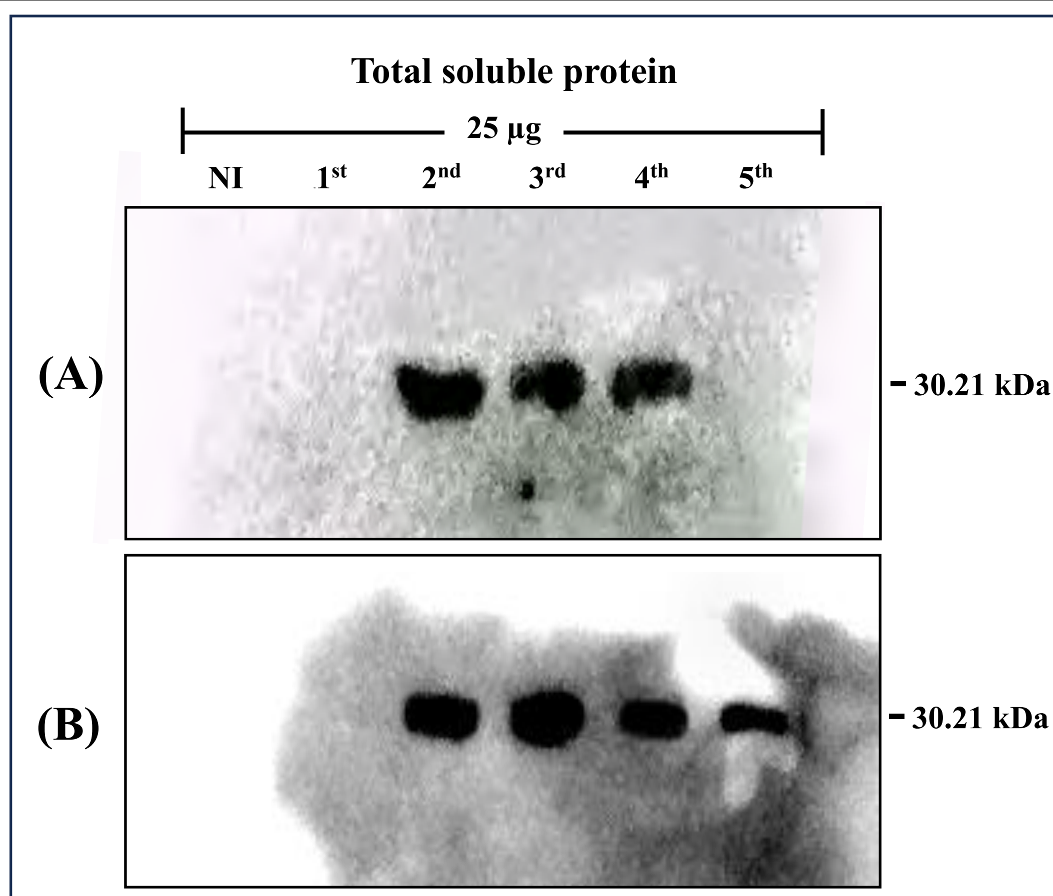



**Figure 5.8: Confirmation of OmpK protein in infiltrated leaves. (A)** Samples of *Spinacia oleracea* L. (F1 hybrid) **(B)** Samples of *Lactuca sativa* L. (Grand Rapid) M: 1 kb DNA marker; NI: Non-infiltrated leaves; 1-5: Infiltrated leaves collected on different days.

### 5.3.5. OmpK protein quantification via ELISA

Quantification of transgenic protein in all collected infiltrated leaves ($1^{st}$-$5^{th}$ day) was conducted via ELISA to determine which day infiltrated leaf exhibited higher expression level of OmpK by following Verma *et al*. (2008). For this purpose, a series of dilutions of total soluble protein isolated from infiltrated and non-infiltrated spinach and lettuce leaves were prepared. The estimation of OmpK protein in transgenic plants was conducted by comparing it dilution series of OmpK protein ordered from the company. The OmpK protein maximum expression calculated was 0.35% for spinach and 0.45% for lettuce of TSP on $3^{rd}$ day. For spinach on the $5^{th}$ day, no expression level for OmpK was observed whereas 0.15% of OmpK protein of TSP was produced in the case of lettuce. Figure 5.9 graphically is the transgenic protein percentage in total soluble protein extracted from infiltrated leaves of spinach and lettuce.

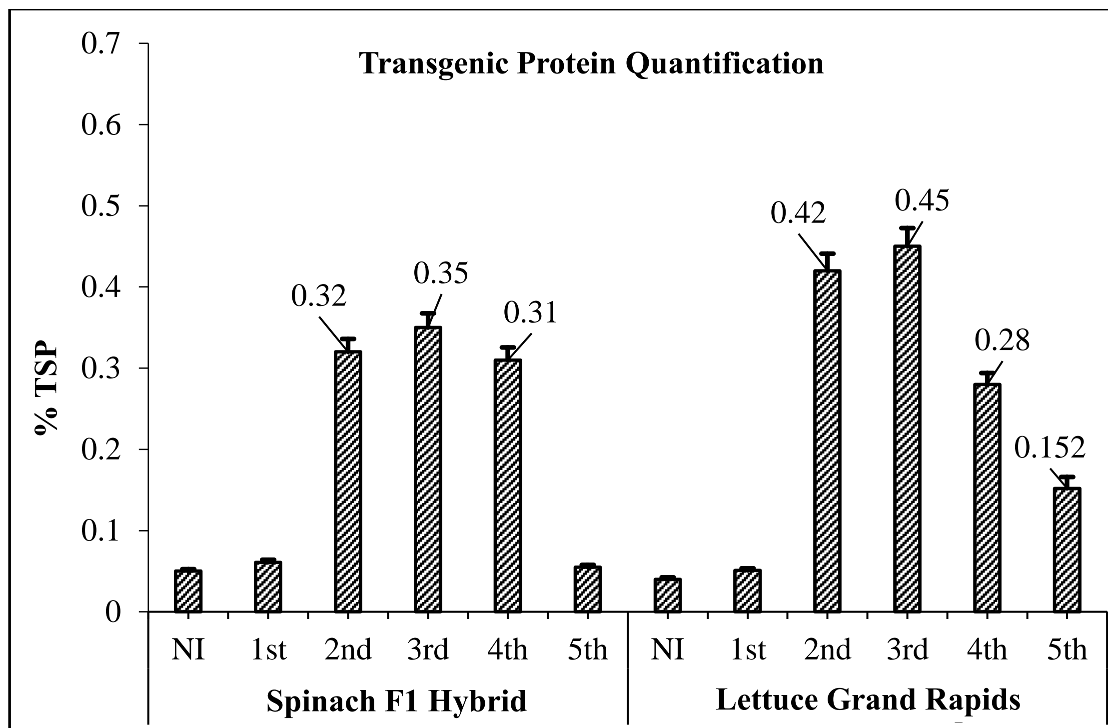



**Figure 5.9: Quantification of OmpK protein in infiltrated leaves samples of *Spinacia oleracea* L. (F1 hybrid) and *Lactuca sativa* L. (Grand Rapid).** M: 1 kb DNA marker; NI: Non-infiltrated leaves; 1-5: Infiltrated leaves collected on different days.

**Annexure 5.1**

**YEB medium for *Agrobacterium* growth for infiltration**

| Components | Concentration |
|---|---|
| Beef extract | 5.0 gm/L |
| Magnesium sulfate heptahydrate | 0.5 gm/L |
| Peptone | 5.0 gm/L |
| Sucrose | 5.0 gm/L |
| Yeast extract | 1.0 gm/L |
| pH | 7.0 |

**Annexure 5.2**

**Infiltration media**

| Components | Concentration |
|---|---|
| Acetosyringone (Stock solution) | 5 mL |
| D-glucose | 250 mg |
| MES (Stock solution) | 5 mL |
| Sodium phosphate tribasic dodecahydrate (Stock solution) | 5 mL |
| Distilled water | ~35 mL |
| Total | 50 mL |

**Annexure 5.3**

**Stock solutions for infiltration media**

| Media | Ingredients | Concentration |
|---|---|---|
| **Acetosyringone (1 M)** | Acetosyringone | 0.196 gm |
| | DMSO | 1 mL |
| **MES (500 mM)** | MES | 4.88 gm |
| | Water | 50 mL |
| **Sodium phosphate tribasic dodecahydrate (20 mM)** | $Na_3PO_4.12H_2O$ | 0.5 gm |
| | Water | 50 mL |

# DISCUSSION

Chapter 6

The present study demonstrates the first report on the expression of OmpK vaccine antigen in higher plants against vibriosis, potentially targeting multiple *Vibrio* species due to the conserved nature of OmpK. The vaccine construct was evaluated for its immunogenic potential and other properties using bioinformatics tools. We chose the tobacco plant for stable expression of OmpK, due to its high biomass, scalability, and potential for high foreign protein expression. The OmpK was expressed via *Agrobacterium*-mediated transformation. The plant-derived vaccine showed significant immunogenicity in mice via oral and subcutaneous routes. The goal of the present study was also to develop a plant-based edible vaccine against vibriosis. For this purpose, the edible plants, (*Spinacia oleracea* L. and *Lactuca sativa* L.) were transiently transformed with the OmpK gene via *Agrobacterium*.

### 6.1. OmpK as a vaccine candidate against vibriosis

*Vibrio anguillarum* and other *vibrio* species, causing vibriosis, pose a significant risk for the aquaculture industry around the world. Several reports are there which show that the frequent overuse of antibiotics in aquafarms to treat infections can give rise to antibiotic-resistant bacteria (Adel *et al*., 2017; Cabello *et al*., 2013; Igbinosa, 2016; Letchumanan *et al*., 2016; Loo *et al*., 2020; H. N. K. Nguyen *et al*., 2016; B. M. Rao & Lalitha, 2015; Scarano *et al*., 2014; Singh *et al*., 2022; Xing *et al*., 2020; Y. Xu *et al*., 2017; N. Yang *et al*., 2021; Yilmaz *et al*., 2022; Z. M. Zhu *et al*., 2018). This poses challenges in the treatment of vibriosis which can be avoided using subunit vaccines. Subunit vaccines consist of specific protein structures from bacteria, focusing on immunogenic non-toxic epitopes to eliminate adverse reactions caused by unrelated epitopes**.** Outer membrane proteins (OMPs) are highly immunogenic due to their exposed epitopes on the surface and thus are promising potent vaccine candidates. Host defence systems can easily identify fractions of the OMPs as foreign antigens and play an important role in eliciting the host immune response (Frans *et al*., 2011; Hamod *et al*., 2012; Ji *et al*., 2020; Khushiramani *et al*., 2012; S.-Y. Wang *et al*., 2003). NCBI-based OMP gene search has filtered out OmpK, OmpU, OmpW, and TolC as highly conserved proteins through comparative analysis among different *Vibrio* species (Y. Li *et al*., 2010; Lu *et al*., 2014; Mao *et al*., 2011).

Outer membrane protein K (OmpK) is a protective antigen against fish vibriosis. OmpK exposure to the external environment is an important characteristic that makes it an effective candidate for the development of a broad-spectrum vaccine (De Groot *et al*.,

2002; Ellis & Kuehn, 2010; Qian *et al*., 2008; C. Zhang *et al*., 2008). OmpK has been reported to have the ability to elevate the level of antigen-specific antibodies, innate immune response (IL-1β, TLR5M, and IL-12p40), cytokine production (IFN-γ and T-bet), and T cell numbers (CD4–1, CD4–2, and CD8α) in vaccinated fish (Hamod *et al*., 2012; S. H. Lee *et al*., 2021; Mao *et al*., 2007). Xu *et al* (2019b) have investigated that OmpK as a DNA vaccine can induce both cellular and humoral immunogenic responses in fish. Hamod *et al*. (2012) expressed the OmpK gene from *V. anguillarum* in *Escherichia coli* as a subunit vaccine with RPS 67.8% in carp. These properties support the use of OmpK as an ideal plant-based vaccine candidate to combat the pandemic or endemic-causing pathogenic *Vibrio* species in aquaculture.

### 6.2. *In silico* characterization of OmpK

As an innovative technological approach, *in silico* designing of vaccines, using bioinformatics tools and biological databases has allowed the accurate prediction of vaccine properties and the development of highly efficient and safe vaccines (Baliga *et al*., 2018). In the present study, we used a reverse vaccinology approach guided by immune informatics to investigate the potential of OmpK as a potential vaccine. In this regard, prophylactic vaccine design followed by *in silico* analysis of the *V. anguillarum* target gene OmpK against vibriosis is a promising strategy, as it can not only save researchers from costly trial-and-error experimental failures but also reduce the costs associated with vaccine design by providing a clear path forward (Ji *et al*., 2020; H. Xu *et al*., 2019a). In our study, a total of 269 amino acid residues (OmpK along with 6x Histidine residues) were used in the generation of the OmpK vaccine construct (OVC).

#### 6.2.1. Epitope prediction in OVC

The immune response would be robust when all CTL, HTL, and linear B-cell epitopes are identified. Several studies reported that for the elimination of infections, a strong T-cell response is required. So, such a vaccine should be invented which can produce robust long-term memory through T-cell activation. Cytotoxic T-cells (CTLs) and Helper T-cells (HTLs) are the most important part of adaptive immunity to make antibodies (Alberts *et al*., 2002; Channappanavar, Fett, *et al*., 2014; Channappanavar, Zhao, *et al*., 2014; R. Chen *et al*., 2021; Janice Oh *et al*., 2012; Samad *et al*., 2022). The recommended web server Immune Epitope Database Nathan EL 4.1 was used to find the most immunodominant MHC-I binding CTLs epitopes and MHC-II binding

HTLs epitopes in the OVC. The IEDB MHC-I is a reliable and freely accessible online tool that has vast applications in the field of bioinformatics and biomedical research (Fleri *et al*., 2017). A total of ten MHC-I binding and five MHC-II binding epitopes were detected in our OVC.

Effective vaccination against measles and rubella reflects the significance of protective antibodies. Defence against diseases depends on the neutralizing antibodies simulation in addition to adaptive immunity elicited by the T-cells. CTLs can only kill infected cells, whereas elimination of infected cells is carried out by antibodies and prevents infection due to infectious agents. The process is called neutralization (Dörner & Radbruch, 2007; Hsueh *et al*., 2004; Janice Oh *et al*., 2012). Linear B-cell epitopes in the full-length primary protein sequences were predicted by ABCpred. This server is based on an artificial neural network (ANN) and recurrent neural network to predict B-cell epitopes with 65.93% accuracy. Additionally, the server provides users with the flexibility to choose the length of the window from a range of window length options of 10, 12, 14, 16, and 20 (Saha & Raghava, 2006, 2007). Based on *in silico* analysis, five B-cell domains were detected in OVC. Given that both cellular and humoral immune responses are essential against infection (Oh *et al*., 2012), epitope analysis predicted OVC as a potent vaccine candidate.

### 6.2.2. OVC's 2-D structures and physiological parameters

Various physiochemical characteristics such as toxicity, allergenicity, antigenicity, amino acid composition, molecular weight, theoretical isoelectric point (pI), atomic composition, expected half-life in vitro and in vivo, grand average of the hydropathicity (GRAVY) value, extinction coefficient, instability index (II), and aliphatic index of the two-dimensional structure of our vaccine construct were assessed by processing the OVC sequence with the help of online web servers such as ToxinPred, AllerTOP v2.0, and VaxiJen v2.0, ExPASy Protparam, and SCRATCH Protein Predictor.

Alpha-helix and unfolded protein regions are significant structural forms of antigen that can be identified by antibodies, produced as a result of infection, to elicit immunogenic responses (Shey *et al*., 2019). The predicted secondary structure of the OVC consisted of 44.61% (random coil), 28.62% (extended strand), 21.93% (alpha helix), and 4.83% (beta turn), which indicated OVC as a suitable vaccine candidate. There are many therapeutic proteins which are highly versatile, specific, and less toxic. But low

solubility, non-immunogenicity, proteolytic degradation, physiochemical instability, and short half-life are some disadvantages that have been observed (Antosova *et al*., 2009; Bruno *et al*., 2013; Di, 2015; Kardani *et al*., 2020). The theoretical isoelectric point (PI) of OVC was 5.64 indicating that the vaccine was acidic. The instability index value of less than 40 suggests that the protein is highly stable to facilitate the onset of immunogenic responses (Enayatkhani *et al*., 2021). OVC was classified as a stable protein according to the instability index value, which was 12.88, but insoluble following over-expression. The OVC aliphatic index (65.99) showed that it is stable at various temperatures.

Vaccine candidates should be non-allergen, highly antigenic, and non-toxic. Proteins which can elicit IgE antibodies are regarded as allergens. So, safe and effective vaccines should not be allergenic (Dimitrov *et al*., 2014; Enayatkhani *et al*., 2021). Furthermore, a good vaccine must generate memory cells against pathogenic epitopes as most peptide-based vaccines have low immunogenicity. VaxiJen, webserver, determines antigenicity of antigens by converting protein into a set of essential amino acid components and AllerTOP v. 2.0, webserver, predicts recombinant protein allergenicity based on residue hydrophobicity, size, abundance, helicity, and β-strand formation propensity. Both servers are based on the Automated Cross-Covariance (ACC) algorithm. Compared to many allergy prediction methods, AllerTOPv.2 has the highest accuracy (88.7%) (Dimitrov *et al*., 2014; Doytchinova & Flower, 2007, 2008; Kardani *et al*., 2020). Based on these software predictions, OVC was non-allergen and highly antigenic.

Toxins, natural or synthetic, have the potential to induce adverse health effects or diseases in an individual on exposure. Toxicity represents a significant challenge in protein/peptide-based therapy (Rathore *et al*., 2023; N. Sharma *et al*., 2022). Very limited attempts have been made to develop a tool which can detect peptide toxicity. Chaudhary and colleagues based on experimentally validated peptide toxins developed an *in silico* tool, which can predict a toxin based on features like binary profile, hemolytic motifs, and residue-based composition of peptide. Chaudhary and colleagues based on experimentally validated peptide toxins developed an *in- silico* tool, which can predict a toxin based on features like binary profile, hemolytic motifs, and residue-based composition of peptide (K. Chaudhary *et al*., 2016; Kardani *et al*., 2020). ToxinPred is an efficient online tool that enables the prediction of the toxic potential of

peptides, and the screening of non-toxic peptides (Gupta *et al*., 2013; Rathore *et al*., 2023; N. Sharma *et al*., 2022). OVC was found non-toxic. Based on its expected physicochemical properties and high scores across all criteria, this OVC appeared as a potent vaccine to fight vibriosis.

### 6.2.3. OVC's 3-D structure and molecular docking

The 3-D structure was predicted and refined by the Galaxy Refine server. Galaxy refines the model by molecular dynamics simulation. Model 2 among five generated models by Galaxy Refine showed one of the best performances in improving protein structure quality. Further analysis of the OVC-Model 2 was made using the ERRAT quality factor, Ramachandran plot, and Z-score, which predicted the good performance of OVC.

Ramachandran plot calculates torsional angles residue-by-residue in protein and indicates whether residues are in allowed, favoured or outlier regions (Oberholser, 2010). In case of OVC, 86.3% of the residues were in the most preferred regions, and only 1.3% were in disallowed regions. ProSA web based on atomic coordinates detects errors in the software-generated 3D model of protein. ProSA web determines the Z-score (overall model quality) and creates a plot of protein residues residue based on energies. The Z-score values within the acceptable range for protein are -10 to 10 (Enayatkhani *et al*., 2021; Wiederstein & Sippl, 2007). For OVC the Z-score (-5.62) was in an acceptable region.

Using three-dimensional models, researchers can foresee the presence of discontinuous B-cell epitopes, which are necessary for vaccine-induced immune system activation. In the eradication of infections, a critical role is played by interaction between antibodies and epitopes of antigen (Enayatkhani *et al*., 2021). The online server (ElliPro) projected many discontinuous B cell epitopes, pointing to the promising potential of the designed OVC for immunological activation. ElliPro employs Thornton's method to predict discontinuous B-cell epitopes (Jaiswal *et al*., 2020; Ponomarenko *et al*., 2008; Ramana & Mehla, 2020).

In both the invertebrate and vertebrate lineages, a crucial role is played by Toll-like receptors (TLRs) in their defence against invading pathogens. Activation of subsequent signalling pathways occurs upon recognition of ligands by respective TLRs (Akira & Takeda, 2004; Fitzgerald & Kagan, 2020; Medzhitov, 2001; Purcell *et al*., 2006; Sahoo,

2020). Studies have shown that the regulation of TLR-5 paralogs in fish is differentially regulated in response to bacterial infection and ligand stimulation in the tissues. Soluble TLR-5 participate in host defence against microbes in humans as well as in fish (Z. Dong *et al.*, 2023; F. Gao *et al.*, 2022; S. D. Hwang *et al.*, 2010; Tsujita *et al.*, 2006). Thus, in our study, we examined the interaction of the suggested vaccination construct with the TLR-5 by performing docking analysis using a web server ClusPro 2.0. The docking analysis indicated sufficient and meaningful contact between the vaccine design and the immune cell receptor.

To determine the best orientation between OVC and TLR-5 docked complex we utilized protein-protein molecular docking via PatchDock webserver. This web server focuses on recognizing and matching patterns on protein surfaces in the most favourable conditions. Afterwards, the top 10 best-docked models were refined by the FireDock webserver. FireDock algorithm refines the docked structures through soft rigid-body optimization and side-chain rearrangement and ranks them based on various interaction (attractive, repulsive, short-range, and long-range) docked protein residues. All are summed up as binding or global energy of docked complexes (Enayatkhani *et al.*, 2021). We chose the best-docked model based on global energy.

### 6.3. OmpK expression in plants

Few vaccine formulations, such as bacteria killed by formalin or inactivated by heat, have been proven to be protective against vibrio infections. However, whole cell-based vaccines (WCV) may cause adverse effects in vaccine recipients on parenteral administration. Whereas oral administration of WCV frequently requires high doses and produces short-term immunity. Additionally, WCVs have a poor capacity to elicit cellular immune responses (Hamod *et al.*, 2012; Newman, 1993; Pace *et al.*, 1998; M. Qin *et al.*, 2022). As previously said vaccines should be safe, effective, and able to provide long-term protection against bacterial diseases. A vaccine that can effectively curb the existing situation of vibriosis has not yet been available in the market. So without an efficient vaccine, controlling the spread of vibriosis is difficult (Ji *et al.*, 2020; N. Li *et al.*, 2010; Y. Li *et al.*, 2010; H. Xu *et al.*, 2019a, 2019b). Furthermore, fish vaccines are required on a large scale and this problem can be solved by plant-based production platforms. To control many diseases of aquaculture, vaccination is an effective strategy. In many parts of the world, for a variety of aquatic species, *V. anguillarum* has been identified as a major causative agent. Few studies report the

expression of OmpK in prokaryotic expression systems. The antigens OmpK and LamB have been recently expressed in microalgae (*Nannochloropsis*. sp.) and duckweed, respectively, against vibriosis as vaccine candidates (Abidin *et al*., 2021; Heenatigala *et al*., 2020). However, to date, there has been no study on the expression of OmpK as a vaccine antigen against vibriosis in higher plants.

### 6.3.1. Tobacco as a model plant

A plant-based expression system is an attractive, cost-effective, scalable, and safe platform for protein expression. The transformed plants can be grown easily at a site where the product is needed, and it can be administered in the form of partially processed or unprocessed material (such as plant leaves or the whole plant) orally, subcutaneously, or by intra-peritoneal means (Saba *et al*., 2020; Waheed *et al*., 2011). Most vaccines available in the market are quite costly since these are made using expensive fermenter-based production facilities. The price of a cooling chain and its maintenance, as well as the cost of injection delivery, can be substantial. Vaccines against diseases must be produced efficiently so that they can be made widely available at a low price (Ji *et al*., 2020; H. Xu *et al*., 2019a, 2019b). Thus, an effective and safe vaccine needs to be developed to control vibriosis via the plant-based method.

Tobacco is a non-feed/non-food plant, and it is easily cultivated and transformed. It also has high biomass and an established cell culture system. In the past few decades, extensive research on tobacco characterized it as a model plant for chloroplast transformation because it has a well-characterized genome (Tusé *et al*., 2015). The use of *Nicotiana tabacum* as a model plant for vaccine production is very viable due to the ease of genetic manipulation, high biomass and simple growth requirements (Ruf *et al*., 2001).

### 6.3.2. Hygromycin optimization for the stable nuclear transformation of tobacco

In the present research, we optimized the hygromycin concentration for explants of tobacco as it was not previously established in our Lab. The explants of tobacco were successfully transformed by the GV3101 strain of *Agrobacterium tumefaciens* containing the OmpK gene in plasmid pGWB5.

Tissue culture, plant breeding and genetic engineering are important techniques to attain improvements in plant yield and varieties. These techniques can be used to improve and add target characteristics to existing cultivars. Establishment of effective

protocol is a prerequisite for plant transformation and regeneration via genetic engineering (Brar & Khush, 2021; Bridgen *et al*., 2018; Gulzar *et al*., 2020; Loyola-Vargas & Ochoa-Alejo, 2018; Tazeb, 2017). Regeneration of tobacco has been previously reported via somatic embryogenesis of leaves at 25 and 30 mg/L of hygromycin (Heidari-Japelaghi *et al*., 2020; Kati & Luthar, 2013; Pathi *et al*., 2013). Tobacco regeneration from callus, stem, and leaf at 50 mg/L hygromycin with maximum regeneration efficiency in stem and lowest in leaves (10%) was also documented (Rachmat & Maulana, 2021). Krügel *et al*. (2002) used different concentrations (20, 25, 35 mg/L) of hygromycin at different stages of their study. Another research group also utilized double concentrations of hygromycin (20 and 50 mg/L) to get transformants (Sustiprijatno *et al*., 2022).

In our study, the different concentrations of hygromycin (20, 30, and 50 mg/L) were used to optimize its concentration for the nuclear transformation of tobacco. Explants of tobacco showed resistance up to 20 mg/L of hygromycin, above which explants either became pale or did not develop any callus even after one month. Thus the effective concentration of hygromycin used for the selection of transformed cells was 30 mg/L which was also optimized by Pathi *et al*. (2013) in their study to obtain transgenic explants.

### 6.3.3. Stable nuclear expression of OmpK in tobacco

We transformed tobacco via *Agrobacterium*-mediated transformation to stably express the OmpK antigen in this study. Tobacco was chosen for the OmpK protein expression because of its high totipotency, high biomass, scalability, eukaryotic post-translational modifications, and well-established tissue culturing and transformation protocols (Jube & Borthakur, 2007; Lössl & Waheed, 2011).

We also carried out the expression analysis of OVC. The sequence of the planned vaccine construct should be codon optimized according to the expression system into which it was to be introduced for high-level expression before it can be used in in vitro and in vivo experiments (Atapour *et al*., 2021). Therefore, the OVC sequence was optimized for the eukaryotic plant's codons. The total GC percentage of the OVC was 41.02%. This astoundingly high GC content value was potentially indicative of high protein expression in the host. Evidence suggests that high GC content increases protein transcription rate in animals and plants due to B-Z transition ability and flexibility

provided by GC-rich regions favours open chromatin conformation as compared to AT-rich regions (Goulet *et al*., 2022; Weng *et al*., 2011).

A total of six independent transgenic lines of tobacco expressing OmpK were generated through *Agrobacterium*-mediated transformation. Confirmation of transgene in the nuclear genome of tobacco was carried out by PCR. Whereas, to determine the copy number of transgene in transgenic tobacco qRT-PCR was performed. The maximum integrated copy number was found to be two. The number of copies of the integrated transgene varies when carried out by *Agrobacterium*-mediated transformation as it randomly integrates the transgene into the plant's nuclear genome. However, the average number of copies inserted into the genome is usually 1–2 (L. Wen *et al*., 2012).

We confirmed the expression of OmpK in the leaves of transgenic tobacco via Western blot. The OmpK band equivalent to 30.21 kDa (monomeric form) was observed in transgenic lines. Mao *et al*. (2011) detected the recombinant OmpK of 36 kDa rather than the expected 29 kDa size, which may be due to hyper-glycosylation of Asn-Gly-Ser residues. Such hyper-glycosylation is frequently reported in the proteins expressed via the yeast expression system. In our case protein size was more than 29 kDa because of the six His-tag residues attached to OmpK protein at the N-terminal for detection via western blotting and ELISA. Similarly in another report, OmpK (28 kDa) was expressed in *E. coli* DH5α as a vaccine against vibriosis in Orange-spotted groupers (*Epinephelus coioides*) (N. Li *et al*., 2008).

For the development of vaccines, an adequate amount of transgenic protein is required to meet the optimum required dosage (Saba *et al*., 2020). In the current study, the maximum expression level of OmpK protein was about 0.38% of total soluble protein (TSP) in our transgenic lines L5 and L6. Recombinant protein expression level in stable nuclear expression in plants is low because of random integration as it may cause gene silencing (Burnett & Burnett, 2020).

### 6.3.4. OmpK elicited immunogenic response during animal trials

For the aquaculture industry, there is a need for oral vaccines as a convenient and ideal mode of vaccination, so that the fish can be protected from the stress that it encounters through other methods of vaccination. Studies have shown that immunity in fish can be achieved via oral routes. For the manufacturing of immunogenic proteins as oral vaccines, plant-based production platforms are well-established and promising

biotechnology-based bioreactors (Embregts & Forlenza, 2016; J. K. Ma *et al*., 2013). The transformed plants can be administered in the form of partially processed or unprocessed material (such as plant leaves or the whole plant) orally, subcutaneously, or by intra-peritoneal means (Saba *et al*., 2020; Waheed *et al*., 2011).

Oral vaccines can induce immunity at both the mucosal level and systemic immune level. Oral vaccines can be a better option to counter the pathogens that infect the organisms via mucosal routes such as *Vibrio* sp. as compared to injectable vaccines. Immunization by feeding the plant biomass is of particular interest as it is easy to use and less costly. There have been studies in which the immunogenic potential of plant-expressed vaccine antigens was tested by feeding transgenic tobacco plant material to mice and fish (Cho *et al*., 2018; S. H. Lee *et al*., 2021; Ruhlman *et al*., 2007; Saba *et al*., 2020). In the current study, the immunogenic potential of transgenic tobacco expressing the OmpK antigen was investigated in mice and fish. Mice were orally and subcutaneously immunized by giving the OmpK protein. The results showed that oral delivery and subcutaneous delivery of OmpK induced humoral response in mice.

In fish, oral immunization resulted in a significant immunogenic response. Few previous studies on OmpK as an antigen produced via the prokaryotic expression system also showed immunogenicity in animal models, especially in fish. For oral delivery, one of the main hurdles is the denaturation and degradation of antigens by the stomach's strong acidic environment (Embregts & Forlenza, 2016; N. Li *et al*., 2008; Saba *et al*., 2020; C. Zhang *et al*., 2008). However, in plant-based expression, the encapsulation provided by plant cells could help the antigens bypass the harsh acidic environment of the digestive tract and can elicit the humoral response via the gut epithelium (K.-C. Kwon & Daniell, 2015). The current research is a significant scientific contribution to the limited existing vaccine research against fish diseases and holds a strong potential to develop a vaccine against vibriosis for use in aquaculture, due to the reason that a significant immune response in mice and fish was achieved by plant-expressed protein.

### 6.4. OmpK as an edible vaccine

In this study, we also explored the possibility of developing a plant-based edible vaccine against vibriosis because the plant expression system is an attractive platform for the expression of recombinant proteins. Vaccination has a key role in controlling

many infectious diseases. The research on the development of plant-based vaccines needs to be expanded for the development of oral subunit vaccines against different deadly diseases in edible plants to express a sufficient level of foreign antigenic protein to meet the suitable dosage required for immunization (Chan *et al*., 2016; Okay & Sezgin, 2018; M. Yang *et al*., 2018). The manufacture of vaccines using a plant-based system has many benefits over traditional techniques. Edible plants can provide biotechnological solutions for the creation of extremely safe, quick, high-yield, and affordable vaccinations. Other important characteristics of plant-based edible vaccines are the minimal need for a cooling chain and cost-effective downstream processing (Cardi *et al*., 2010; Saba *et al*., 2019).

Lettuce is a good option to produce recombinant proteins to be used as an oral vaccine because it contains no harmful substances and high biomass. Additionally, recombinant protein purification is easy as it contains fewer substances that can impede the purification. Different studies revealed that oral administration of leaves of edible plants (lettuce) expressing antigen as a vaccine can provide sufficient immunogenicity (Haleh *et al*., 2005; Honari *et al*., 2012; Nakahira *et al*., 2021; Pniewski *et al*., 2011). Dahl (2016) transiently expressed outer capsid proteins (Sigma-1 and Mu-1) from Piscine Reovirus (PRV) in lettuce and tobacco via *Agrobacterium*-mediated transformation to be used as a vaccine in aquaculture.

Spinach is a favourite, nutrient-rich, and popular vegetable around the world (D. V. Cao *et al*., 2017). Reports have shown that DNA delivery in leaves of plants such as red beetroot, spinach, sunflower, and tobacco via *Agrobacterium*-mediated infiltration process has resulted in a maximum yield of up to 50% of TSP in a 4-5 days period (Bolaños-Martínez *et al*., 2022). Reports indicate that a significant level of antibodies has been detected in volunteers who have been fed with transgenic spinach expressing antigens against rabies virus (Naseri *et al*., 2019). In another study chimeric construct of the VP1 gene of the foot and mouth disease virus was transiently expressed via the *Agro*-infiltration method in spinach with a high level of gene expression (Habibi *et al*., 2014). To date, no expression of antigenic protein has been documented in spinach either stably or transiently against fish diseases.

The expected costs linked with the production and delivery of plant-based vaccines are much lower as compared to expressed recombinant proteins in bacterial, mammalian or insect cells. The major issue regarding the delivery of oral vaccines is the denaturation

and degradation of the vaccine in the digestive system (K.-C. Kwon & Daniell, 2015; C.-W. Liu *et al*., 2007). However, bioencapsulation of vaccine antigens within plant cells could sustain an acidic environment of the stomach and reach the gut where it could be absorbed through the gut epithelium (K.-C. Kwon & Daniell, 2015). Plant-based edible vaccine technology is at an early stage for fish vaccines (Bedekar & Kole, 2022; Shin *et al*., 2013).

### 6.4.1 Transient expression of OmpK in edible plants

In this study, we also explored the possibility of developing a plant-based edible vaccine against vibriosis. OmpK gene was transiently expressed in *Spinacia oleracea* L. F1 hybrid and *Lactuca sativa* L. Grand rapid via *Agrobacterium*-mediated transient transformation. PCR confirmed the integration of recombinant Ti plasmid within the plant nuclear genome and the expression of recombinant protein was confirmed via Western blotting. A significant amount of protein was expressed in spinach and lettuce plants.

Transient expression of recombinant protein by the introduction of T-DNA into plants via Agrobacterium is a highly efficient method and results in higher expression of recombinant protein (up to 1,000 folds) in comparison to stable nuclear expression. This method yielded around 1.5 g of recombinant protein per Kg of fresh leaf (Burnett & Burnett, 2020). The important leafy vegetables *S. oleracea* L. and *L. sativa* L. can be grown all over the world (Mane *et al*., 2015; C. Xu *et al*., 2017). In our investigation, we infected spinach and lettuce leaves with the *A. tumefaciens* GV3101 strain, which offered a strong expression of OmpK. According to Sparkes *et al*. (2006), in the case of agroinfiltration of leaves, the expression only requires a few days (Shamloul *et al*., 2014).

In our study, the leaves of both edible plants were infiltrated for five days. PCR analysis showed in the case of spinach the gene expression level diminished on the fifth day whereas in the case of lettuce, no significant difference was observed. Western blotting confirmed the presence of transgenic OmpK protein (30.21 kDa) on the 2$^{nd}$ to 5$^{th}$ days in the case of lettuce whereas only for the 2$^{nd}$ to 4$^{th}$ day in spinach. ELISA analysis revealed that the expression level of OmpK was maximum at 3$^{rd}$ day in both plants (Spinach: 0.35% and Lettuce 0.45%). Following the agroinfiltration, the infiltrated gene expression level usually increases within 3-4 days then dramatically due to activation

of the plant defence system (post-transcriptional gene silencing (PTGS)). PTGS is the anti-viral system of plants and has a crucial role in maintaining genome stability (D. V. Cao *et al*., 2017).

Despite many benefits of edible plants as vaccines including that these can be taken raw and have no need for a purification process, there are strict regulatory requirements for their use as vaccines. This is because edible vaccines could not be administered safely without substantial processing and formulations to ensure consistency in dosage and potency in all products. This dose problem can be overcome by freeze drying of plant material. But still, there is hope because an edible vaccine (maize and potatoes) targeting *E. coli*, has progressed to clinical trials (Phase I) (Burnett & Burnett, 2020; Rybicki, 2010; Shahid & Daniell, 2016).

# *CONCLUSION AND FUTURE PERSPECTIVES*

## Conclusion and Future Perspectives

The present study reports the successful expression of OmpK antigen in tobacco plants via stable nuclear expression. Significant immune response in mice and fish was achieved by plant-expressed protein. The current research is a significant scientific contribution to the limited existing vaccine research against fish diseases and has a strong potential to develop a vaccine against vibriosis for use in aquaculture. The OmpK antigen was transiently expressed in two edible plant species: spinach and lettuce. A significant amount of OmpK protein accumulated in the edible plants. Taken together, this data could help in the development of a cost-effective antigen-based subunit vaccine against vibriosis, with the possible potential of oral delivery. Further, OmpK stable expression in edible plants can be done along with studying the humoral immune response in fish elicited via oral immunization.

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*Chapter 7*

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# In silico designing and characterization of outer membrane protein K (OmpK) from *Vibrio anguillarum* and its expression in *Nicotiana tabacum* for the development of a plant-based vaccine against fish vibriosis

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ABSTRACT

Vibriosis is caused by *Vibrio anguillarum* in various species of aquaculture. A novel, secure, and stable vaccine is needed to eradicate vibriosis. Here, for reverse vaccinology and plant-based expression, the outer membrane protein K (OmpK) of *V. anguillarum* was chosen due to its conserved nature in all *Vibrio* species. OmpK, an ideal vaccine candidate against vibriosis, demonstrated immunogenic, non-allergic, and non-toxic behavior by using various bioinformatics tools. Docking showed the interaction of the OmpK model with TLR-5. In comparison to costly platforms, plants can be used as alternative and economic bio-factories to produce vaccine antigens. We expressed OmpK antigen in *Nicotiana tabacum* using *Agrobacterium*-mediated transformation. The expression vector was constructed using Gateway® cloning. Transgene integration was verified by polymerase chain reaction (PCR), and the copy number via qRT-PCR, which showed two copies of transgenes. Western blotting detected monomeric form of OmpK protein. The total soluble protein (TSP) fraction of OmpK was equivalent to 0.38% as detected by ELISA. Mice and fish were immunized with plant-derived OmpK antigen, which showed a significantly high level of anti-OmpK antibodies. The present study is the first report of OmpK antigen expression in higher plants for the potential use as vaccine in aquaculture against vibriosis, which could provide protection against multiple *Vibrio* species due to the conserved nature OmpK antigen.

## 1. Introduction

Aquaculture is the fastest-growing industry and is crucial for the economy around the world. In 2020, global fish production reached 178 million tonnes, which was a slight decline from the recorded milestone of 179 million tonnes in 2018. The steady development in aquaculture resulted in the global production of aquatic animals reaching 87.5 million tonnes in the year 2020 which grew by 2.7% as compared to 2019 (Béné et al., 2016; Clarke et al., 2013; FAO, 2022; Froehlich et al., 2018; Ji et al., 2020). For a sustained and developed aqua-industry, its prevention from diseases is a major challenge due to opportunistic pathogens (Clarke et al., 2013; Kelly and Renukdas, 2020; Yue and Shen, 2021; Żaczek et al., 2020). Vibriosis is a fish disease caused by gram-negative halophilic bacteria, a significant limiting factor for the aquaculture industry. The main causative agents are *Vibrio anguillarum*, *Vibrio Haeveyi*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus* (Heenatigala et al., 2020; Li et al., 2010; Naka and Crosa, 2011). Depending on the strain, the *Vibrio* species' attack can be malignant or non-malignant. Vibriosis results in lethal hemorrhagic septicemia, ulcers, and abscesses on the skin leading to massive mortality in fish species (Frans et al., 2011; Huzmi et al., 2019; Ina-Salwany et al., 2019; Istiqomah and Isnansetyo, 2020; Ji et al., 2020; Uzun and Ogut, 2015).

Only a few antibiotics and vaccines are in the market for aquaculture as compared to livestock diseases (Bondad-Reantaso et al., 2023;

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Endersen et al., 2014; Heuer et al., 2009; Yue and Shen, 2021). Excessive use of anti-bacterial drugs has resulted in the emergence of antibiotic-resistant microorganisms as well as severe environmental and health concerns. Thus, the development of vaccines is an effective solution for the prevention and controlling diseases in aquaculture. Most of the vaccines currently in use come from lab-cultured pathogens through inactivation or attenuation, with the risk of reversion to an active infection. One of the major hurdles in developing vaccines is the variety of bacterial strains including those from the same species (Baliga et al., 2018; Giddings, 2001; Hamod et al., 2012; Heenatigala et al., 2020; Kayansamruaj et al., 2020; Marsian and Lomonossoff, 2016; Mzula et al., 2019).

Gram-negative bacteria are characteristically surrounded by unique outer membrane proteins (OMPs). The immunological defense mechanism of the host can easily identify OMPs as foreign antigens and activate the host's acquired immune response due to their localization. In recent years, for the development of subunit vaccines, the OMPs have been attractive targets due to their high immunogenicity, conserved nature, and exposed epitopes (Hamod et al., 2012; Heenatigala et al., 2020). Outer membrane protein K (OmpK) could be used as a prospective vaccine candidate against vibriosis as several studies have shown its immunogenicity, efficiency, and viability. The use of bioinformatics tools provides an excellent basis for predicting the immunological, physiochemical, and affective aspects of a vaccine construct, and further helps in actual vaccine development. Several modernized webservers for precise immune-dominant component prediction of vaccine candidates have been established (Baliga et al., 2018; Hamod et al., 2012; Islam et al., 2022; Li et al., 2010; Parvizpour et al., 2020; Sunita et al., 2020; Wang et al., 2021). OmpK is a potential candidate for reverse vaccinology (RV) for the identification of its immunogenic determinants, non-toxicity, safety, and eliciting cell-mediated and humoral immunity.

The vaccine administration methods for fish differ depending on species, pathogens, temperature, and environment. There are three different methods for immunizing fish, each with some advantages and disadvantages namely intraperitoneal injections, oral administration of vaccines, or immersion in diluted vaccine solutions (Muktar et al., 2016). Oral vaccination is the ideal route for fish vaccination from an economical point of view, as it is cheap, simple, stress-free, and can be used for mass vaccination of fish of all sizes (Heenatigala et al., 2020; Muktar et al., 2016). There are several advantages of using plant-based system as bioreactors for vaccine production, which make this system ideal alternative for vaccine production as compared to the conventional fermenter-based systems. The plant-based system is cost-effective, safe, and reliable. Some other advantages include: significantly high expression of expressed antigens, ease of manipulation, low production costs as plants are easy to grow anywhere with minimal and inexpensive resources (Lössl and Waheed, 2011). Further, plants can be scaled up to the larger areas using transgenic seeds (act as stable genetic resource), thus increasing the production capacity with less costs, compared to bacterial systems where scaling up is costly due to expensive culture media and maintenance other conditions related to the fermenter-based culture. For oral vaccination, the plant's rigid and thick cell walls may protect the expressing antigen/recombinant proteins from the acidic environment of the foregut to the hindgut by encapsulating them safely and successfully in adequate amounts (Giddings, 2001; Marsian and Lomonossoff, 2016; Rigano and Walmsley, 2005; Saba et al., 2020).

In the present study, potential sites of immunogenicity in the OmpK antigen were identified. A complete sequence of the modified *OmpK* gene was used to develop a vaccine that may protect the fish against vibriosis. Parameters such as physiochemical analysis, antigenicity, immunogenicity, allergenicity, and solubility prediction of the developed construct of OmpK were studied, subsequently followed by its two and three-dimensional structures' generation and verification. Finally, codon optimization and Gateway® cloning of *OmpK* in a pGWB5 vector were performed, followed by its expression via *Agrobacterium*-mediated transformation in *Nicotiana tabacum*. The transgene integration confirmation in the transformed plant was done via conventional polymerase chain reaction (PCR). Transgene copy number was determined via quantitative real-time PCR (qRT-PCR). OmpK antigen's successful and proper expression was confirmed via Western blotting.

## 2. Materials and methods

### 2.1. Retrieval of gene sequence, modification, and vaccine construct

The *OmpK* gene (Gene bank accession no. **FJ705222.1**) was retrieved from the National Center for Biotechnology Information (NCBI). Basic Local Alignment Search Tool for Protein (BLASTp), analysis was performed to determine the level of conservation of the protein among different *Vibrio* strains. The retrieved gene sequence was translated into the amino acid sequences via EMBOSS Transeq tool. The *OmpK* sequence was modified by the addition of six histidine amino acid residues at the C terminal to be used as an OmpK vaccine construct (OVC).

### 2.2. Epitope prediction

Different online tools can be used to predict the immunodominant epitopes that would activate Cytotoxic T cells (CTLs), Helper T cells (HTLs), and B cells. In all the servers used, the OVC sequence was uploaded in FASTA format. The recommended Immune Epitope Database (IEDB MHC-I) Nathan EL 4.1, was used to find the most immunodominant MHC-I binding CTLs epitopes. The default HLA allele reference set was chosen. Linear B cell epitopes were predicted via the ABCpred tool. This server is based on an artificial neural network (ANN) with a 65.93% accuracy. The window length of sixteen and the threshold of 0.8 was set. The overlapping filter was turned off. To measure the affinity binding between MHC-II and epitopes in terms of $IC_{50}$, the webserver "Immune Epitope Database (IEDB MHC-II)" was used. The values $< 50$ nM refer to strong, $< 500$ nM refer to moderate, and $< 5000$ refer to weak binding affinities of epitopes for MHC-II. All the parameters were set to their default values, which were: prediction method IEDB 2.22, HLA-DR, selected length fifteen for OVC, and full HLA reference set for epitope mapping. All the epitopes were also analyzed for toxicity, allergenicity, and antigenicity via different online tools namely: ToxinPred, AllerTOP v2.0, and VaxiJen v2.0, respectively.

### 2.3. Characteristics evaluation of OVC

Physiochemical properties of the OVC, as well as the antigenicity, allergenicity, and toxicity, were checked using different tools. Toxicity, allergenicity, and antigenicity analysis were done for the OVC sequence, using online tools ToxinPred, AllerTOP v2.0, and VaxiJen v2.0, respectively. The physicochemical properties of the OVC such as amino acid composition, molecular weight, theoretical isoelectric point (pI), atomic composition, expected half-life in vitro and in vivo, grand average of the hydropathicity (GRAVY) value, extinction coefficient, instability index (II), and aliphatic index of the vaccine construct was assessed with the help of the tool ExPASy Protparam. The SOLpro of the SCRATCH Protein Predictor tool was used to find out the extent of the solubility of the vaccine construct upon its expression.

### 2.4. Two and three-dimensional structures of OVC

The secondary structure of the vaccine construct was found by the SOPMA server by inserting the OVC amino acid sequence in FASTA format and default settings were selected. The 3-D structure of the OVC was determined by using the GalaxyRefine tool. The resulting models were verified using the ERRAT quality factor and PROCHECK (Ramachandran plots) programs of the UCLA-DOE LAB SAVES v6.0 server and its protein structure Z-score value was checked with the ProSA-Web tool. PyMOL was used to visualize the 3-D structures.

### 2.5. Discontinuous B cell epitope mapping and molecular docking of OVC

Conformational discontinuous B cell epitopes were mapped on the vaccine candidate using the ElliPro tool by submitting a PDB file of the best model of the designed OVC. Molecular docking between the Toll-Like Receptor (TLR) 5 (RCSB PDB ID: 3J0A) (Joshi et al., 2021) of immune cells (receptor), and the designed vaccine (ligand) was carried out by ClusPro 2.0 protein-protein docking server. This server performs rigid body docking using the Fast Fourier Transform (FTT). PatchDock webserver was used to cross-verify the binding affinities between the OVC and the TLR-5 (Schneidman-Duhovny et al., 2005). To FireDock the top 10 results from PatchDock analysis for further improvement and ranking were submitted (Andrusier et al., 2007).

### 2.6. Codon optimization and Gateway® cloning

For actual expression of the OmpK in plants, the sequence of gene was codon optimized using GenSmart™ Codon Optimization according to the plant expression system. This webserver also determines whether a target gene is being expressed in the host system by analyzing its GC content. The *OmpK* gene (Gene bank accession no: **FJ705222.1**) was synthesized from BIOMATIK (Canada) in a pUC57 vector with Histidine-tag (6×His-tagged) at C-terminal to express the *OmpK* transgene in *N. tabacum*. For the construction of the binary expression vector, the Gateway® cloning kit (Invitrogen, USA) was used according to the manufacturer's protocol (Karimi et al., 2002). *OmpK* gene was cloned into pDONR™221 via BP recombination reaction to get the entry vector, pENTR-OmpK. Afterward, the LR recombination reaction using pENTR-OmpK and destination vector pGWB5 a final binary expression vector pEXP-OmpK was obtained. The proper integration of the *OmpK* gene at each step of cloning was verified by PCR with different sets of primers (Data not shown) in the final pEXP-OmpK.

### 2.7. Transformation of Agrobacterium tumefaciens

For the transformation of *Agrobacterium tumefaciens* strain GV3101 with the pEXP-OmpK, Electroporator (BioRad, USA) was used. 60 μL of freshly prepared electro-competent cells were thawed for 10 minutes on ice and mixed with a pEXP-OmpK (2 μL) in the cuvette and then electroporated according to the manufacturer's protocol followed by the addition of SOC media (250 μL). Incubated for 2–3 hours at 28°C with gentle shaking. Then streaked on an LA plate containing kanamycin (50 mg/L) for selection and placed overnight at 28°C.

### 2.8. Agrobacterium-mediated transformation

The seeds of wild-type *N. tabacum* cv. Petit Havana was surface sterilized for 1 minute with 70% (v/v) ethanol solution and rinsed with double distilled autoclave water thrice. Then dried on filter paper. Sterilized seeds were in vitro grown on agar-solidified Murashige and Skoog (1962) medium at 25 °C. *Agrobacterium tumefaciens* containing pEXP-OmpK was used for transforming *N. tabacum* explants. The bacterial culture ($OD_{600}$ 0.6–0.8) was pellet down via centrifugation (3600×g_10 minutes) and then re-suspended in liquid MS media containing acetosyringone (infection media).

Leaves of in vitro grown 2–3 weeks old tobacco plants were cut into 5–8 mm pieces and immersed into infection media (8 minutes). Infected explants were placed on regeneration media (RMOP) for organogenesis of the plant in the dark. After two days, for the selection of transformed plants, the explants were shifted to RMOP media (30 mg/L hygromycin) (Pathi et al., 2013). In vitro transgenic plants were grown under optimum conditions (16 hours light/8 hours dark; 25±1°C) in a growth room and were then acclimatized.

### 2.9. Transgene confirmation via PCR

Genomic DNA from the wild-type and transgenic tobacco plants (100 mg) was extracted via hexadecyltrimethylammonium bromide (CTAB) method as described by Murray and Thompson (1980). To confirm the transformation and integration of the *OmpK* gene in the transgenic plants, PCR was performed by using ThermoFisher Scientific (USA) Taq polymerase standard PCR Kit. 1 μM each of forward and reverse primer and 100 ng of template DNA was used. Integration of the *OmpK* transgenic tobacco genome was confirmed by different sets of primers (Data not shown). The PCR product was resolved on 1% agarose gel stained with 5 ng/mL ethidium bromide. A gel documentation system (FluorChem FC3, USA) was used to visualize bands.

### 2.10. Copy number determination via qRT-PCR

Transgene copy number in the transformed plant was determined through quantitative real-time PCR (MyGo Pro Real-time PCR_Stokesley, Middlesbrough, UK) according to the protocol described by Wen et al. (2012). 20 μL qRT-PCR reaction mixture contained 10 μL SYBR Green (ThermoFisher Scientific, USA), 1 μL of 100 ng template DNA, 1 μM each of forward and reverse primer, and 8 μL of autoclaved distilled water. By using the genomic DNA from the wild-type *N. tabacum* and transgenic lines, the standard curves were determined for both *OmpK* transgene and the endogenous *β*-actin gene. DNA was diluted in series to get 1, 10, 100, and 1000 ng per reaction as final concentrations and qRT-PCR was run with independent repetitions in triplicate.

### 2.11. Western blotting

Transgenic and wild-type tobacco (100 mg) leaves were suspended in 400 μL of protein extraction buffer after grinding in liquid nitrogen to extract total soluble protein (TSP) (Saba et al., 2020). The homogenized sample was centrifuged at 20,000 g at 4 °C for 10 minutes and the supernatant (soluble fraction (SF) of protein) was collected. Total protein concentration in the supernatant was determined against standard (Bovine serum albumin, BSA) via Bradford assay. The TSP (20 μg) was heated with 4X sample buffer (5 μL) at 95 °C for 10 minutes. Protein bands were separated on 15% SDS-PAGE Gel and electro-transferred onto nitrocellulose membrane (ThermoFisher Scientific, USA) for 1 hour at a 110 V in XCell4 Surelock™ Midi Cell (Invitrogen, USA). The blocking of the membrane was done according to the protocol described by Saba et al. (2020). The membrane was allowed to react with anti-His antibody (1:10,000 dilution in TBS-T containing 5% BSA, and 0.05% sodium azide) overnight at 4 °C with gentle agitation. The membrane was washed thrice with PBS followed by incubation with horse radish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (IgG) antibodies (1:10,000 dilution in TBS-T containing 5% BSA) as secondary antibody for 1.5 hours at room temperature. The membrane was washed again thrice with TBS-T. The bands were visualized by dipping the membrane in a Chemiluminescent HRP substrate (Millipore, USA) and detected by a chemiluminescent system (FluorChem FC3, USA).

### 2.12. Quantification of transgenic protein via ELISA

Protein extracts were prepared in extraction buffer from the fresh leaf of transgenic tobacco as described by Saba et al. (Saba et al., 2020). The homogenized sample was centrifuged at 20,000 g at 4 °C for 10 minutes and the supernatant (SF) was collected. This supernatant was further used for the transgenic protein (TP) quantification via ELISA by following the protocol given by Verma et al. (2008). The amount of TP as a percentage of the TSP of transformed leaf material was calculated by the formula:

$$TSP = \left(\frac{TP}{TSP}\right) \times 100$$

### 2.13. Pre-clinical trials in mice and fish

The experiment on mice was conducted at the Primate facility of Quaid-i-Azam University Islamabad, Pakistan, and on the fish experiment in the Lab. BALB/c mice (8–10 weeks old) and *Hypophthalmichthys molitrix* (silver carp; 57.8±1.3 g and 19.4±1.1 cm) were used for the experiment. Mice were divided into seven groups (six members each), labeled as Group A: Normal group with no dose; Group B: PBS solution oral delivery; Group C: PBS solution subcutaneous delivery; Group D: WT tobacco TSP oral delivery; Group E: WT tobacco TSP subcutaneous delivery; Group F: Transgenic tobacco TSP oral delivery, and Group G: Transgenic tobacco TSP subcutaneous delivery. Fish were divided into three groups (ten members each) labeled as Group H: Normal group on commercial feed; Group I: WT tobacco oral delivery mixed in feed pellet, and Group J: Transgenic tobacco oral delivery mixed in feed pellet. For oral and subcutaneous dosing of mice, the dose was calculated by rearranging the formula given by Verma et al. (2008) so that Group F and G would receive 10 μg of transgenic protein by dissolving an appropriate amount of transgenic plant in 500 μL of PBS for oral dosing and 100 μL of PBS via subcutaneous injections. For oral dosing of fish, the protocol described by Shin et al. (2013) was followed, with little modification in dosing pattern, so that each fish could receive 10 μg/10 gm fish body weight of transgenic protein in a feed pellet.

$$\textit{Amount of transgenic protein} = \frac{TP \times V_{PBS/pellet}}{W_{TLM} \times 10^6}$$

TP = Transgenic protein (ng/mL or ng/gm), $V_{PBS/pellet}$ = PBS/pellet volume (mL or gm), and $W_{TLM}$ = Transformed leaf material (grams). The mice were immunized on the 1st, 7th, 14th, and 21st day. To isolate serum from the blood of mice, they were euthanized after one week of the last boost. Fish immunization was done on the 1st, 5th, 10th, and 15th day. Fish blood samples were collected after seven days of last immunization. All animal handling was done per the accepted practices outlined in "Guide for the Care and Use of Laboratory Animals (National Academies Press, Washington)" and approved by the Quaid-i-Azam University's (Islamabad, Pakistan) ethical committee.

### 2.14. Enzyme-linked immunosorbent assay

To determine the level of IgG in the mice's blood serum enzyme-linked immunosorbent assay (ELISA) was performed. Transgenic tobacco TSP (5 μg/well) for 24 hours at 4 °C was coated in a 96-well microtiter plate. The plate was washed three times with TBS-T (TBS containing 0.1% Tween-20) followed by blocking with TBS-TM (TBS containing 0.3% Tween-20 and 3% skimmed milk) for 1 hour at 37 °C. 50 μL of isolated test sera was loaded into each well followed by 50 μL of HRP-conjugated goat anti-mouse IgG secondary antibody (1:10,000) addition. Incubation of the plate was done for 1 hour at 37 °C and washed with TBS-T thrice. 100 μL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (ThermoFisher Scientific, USA) was added to each well. The reaction was stopped by 0.16 M $H_2SO_4$ (100 μL) after 10–20 minutes. Absorbance was recorded at 450 nm with a microtiter plate reader (Bio-Rad, Japan).

### 2.15. Statistical analysis

To perform statistical analysis such as for determining the standard deviations, and drawing graphs Microsoft Excel was used.

## 3. Results

### 3.1. Antigen sequence retrieval and OmpK vaccine construct

From NCBI the *OmpK* gene (Gene bank accession no. **FJ705222.1**) was retrieved. Six histidine residues were added to the sequence at the C-terminal. The translation of gene sequence to amino acid sequence was performed by the EMBOSS Transeq Tool. A total of 269 amino acids were found in the final OVC (Fig. 1A). The BLASTp results showed the conserved nature of the OmpK protein with the conserved domain database ID 225787 belonging to the *Tsx* protein family.

### 3.2. Epitopes prediction

For predicting immune-dominant and safe epitopes for activation of vital immune system cells such as CTLs, HTLs, and B cells, the OVC sequence was examined for MHC-I, MHC-II binding epitopes, and B cell epitopes. MHC-II binding epitopes are crucial for the activation of Helper T cells. The final, antigenic, non-allergenic, and non-toxic MHC-I, B cells, and MHC-II binding epitopes are shown in Table 1.

### 3.3. Characteristics evaluation and physiochemical properties of OVC

The different physiochemical properties of the OVC were examined with the help of the ExPASy Protparam tool, and SOLpro of the SCRATCH protein predictor tool. The theoretical isoelectric point (pI), molecular weight, amino acid composition, atomic composition, expected half-life in vitro and in vivo, instability index, extinction coefficient, Grand average of hydropathicity (GRAVY), aliphatic index, and the solubility of OVC (when overexpressed) were determined (Table 2). Vaccines must not cause any allergic reaction and must not be toxic. Therefore, antigenicity, allergenicity, and toxicity of the whole vaccine construct analysis were also done (Table 2).

### 3.4. Two and three-dimensional structures of OVC

The accuracy of SOPMA's predictions is higher than 80% for secondary structures. For OVC, the SOPMA server projected 21.93% α-helix (h), 4.83% β-turn (t), 44.61% random coils tabulated (c), and 28.62% extended strand (e) (Fig. 1A). GalaxyRefine tool was used to create a 3-D structure for the vaccine construct. The tool predicted five models for OVC. However, "Model 2" (Fig. 1B) was picked based on its quality factor from ERRAT (91.6%), the Ramachandran plot analysis from PROCHECK, and the Z-score (−5.62) values from ProSA-Web. The Ramachandran plot for the selected model of OVC showed that 86.3% of the residues were in the most preferred regions, 11% were in additional allowed regions, 1.3% were in generously allowed regions, and only 1.3% were in disallowed regions (Fig. 1C). All the values have shown that "Model 2" is of good quality.

### 3.5. Detection of discontinuous B cell epitopes

The production of antibodies that are specific to a pathogen, which is an essential step in the development of an immune response, is dependent on the activation of B cells. The discontinuous epitopes for the OVC were found by utilizing the default settings for the ElliPro webserver. The software identified nine discontinuous epitopes for the OVC (Fig. 2).

### 3.6. Molecular docking of OVC

ClusPro 2.0 Protein-Protein Docking server was used for molecular docking analysis of the vaccine candidate against TLR-5 (RCSB PDB ID: 3J0A). Twenty-nine docked structures due to the possible interactions between TLR-5 and the OVC were generated as a result. Further analysis was done with the PatchDock webserver and its refinement and scoring with FireDock. The best-docked model (Fig. 1D) with the lowest energy −1405 was chosen. The values of attractive Van der Waal's forces (−43.82), energy contributed by hydrogen bonds (−2.89), and highest global energy (−57.96) by the docked model were also considered during the selection of the best-docked model.

**Fig. 1.** OVC in silico characteristics. (A) Two-dimensional structure. (B) Three-dimensional structure (C) Ramachandran plot for the “Model 2”. (D) Docked complex of “Model 2” (hot pink) and TLR-5 (marine blue). Interacting amino acids (black), polar contact (black dashes), any contacts within 3.0 Å (yellow dashes), all pi interactions (forest green dashes), and Van der Waal’s forces with distance ratio < 0.75 (orange dashes).

**Table 1**
Predicted MHC-I, MHC-II, and B cells binding epitopes and their characteristics.

| Epitopes binding Predictions | | | | | | | Antigenicity | | Allergenicity | Toxicity | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Allele | Start | End | Length | Epitope Sequence | Peptide Score | Percentile rank | Protective score | Probability | | Toxicity Score | Probability |
| **MHC-I Binding Epitopes** | | | | | | | | | | | |
| HLA-A*23:01 | 255 | 263 | 9 | HYLAVTYKF | 0.995046 | 0.01 | 0.9475 | Antigenic | Non-Allergen | 5.75E-29 | Non-toxic |
| HLA-B*07:02 | 97 | 105 | 9 | APRMSLDAL | 0.993989 | 0.01 | 1.1807 | | | 2.27E-30 | |
| HLA-A*24:02 | 189 | 198 | 10 | SYQGYIDYQF | 0.949531 | 0.01 | 0.8898 | | | 6.73E-13 | |
| HLA-A*23:01 | 174 | 182 | 9 | NWFKPFYFF | 0.935255 | 0.01 | 1.602 | | | 1.89E-19 | |
| HLA-A*31:01 | 91 | 99 | 9 | KIFMKFAPR | 0.879046 | 0.04 | 1.4345 | | | 5.28E-14 | |
| HLA-B*15:01 | 231 | 239 | 9 | GLKGYKDVY | 0.819983 | 0.06 | 0.6834 | | | 5.28E-23 | |
| HLA-B*58:01 | 139 | 147 | 9 | LGSDVNVPW | 0.802094 | 0.12 | 0.8987 | | | 5.94E-13 | |
| HLA-A*03:01 | 227 | 236 | 10 | AVGYGLKGYK | 0.767764 | 0.11 | 1.2182 | | | 5.59E-27 | |
| HLA-B*40:01 | 58 | 66 | 9 | MEFGGRSGI | 0.728265 | 0.14 | 0.7659 | | | 3.67E-18 | |
| HLA-B*07:02 | 96 | 105 | 10 | FAPRMSLDAL | 0.670787 | 0.14 | 1.3956 | | | 3.58E-27 | |
| **MHC-II Binding Epitopes** | | | | | | | | | | | |
| HLA-DRB1*09:01 | 7 | 21 | 15 | ALGLVAATSAPVMAA | ——— | 0.12 | 0.4018 | Antigenic | Non-Allergen | 2.59E-14 | Non-toxic |
| HLA-DRB1*04:01 | 74 | 88 | 15 | DIFNLLSNPSSDKEG | ——— | 0.15 | 0.5595 | | | 3.88E-12 | |
| HLA-DQA1*05:01/DQB1*02:01 | 37 | 51 | 15 | QFNLMAAIDELPGES | ——— | 0.24 | 0.7497 | | | 3.70E-19 | |
| HLA-DRB1*09:01 | 6 | 20 | 15 | LALGLVAATSAPVMA | ——— | 0.3 | 0.5157 | | | 3.59E-13 | |
| HLA-DQA1*01:02/DQB1*06:02 | 11 | 25 | 15 | VAATSAPVMAADYSD | ——— | 0.32 | 0.5207 | | | 1.40E-05 | |
| **B cell Epitopes Predictions** | | | | | | | | | | | |
| ——— | 191 | 206 | 16 | QGYIDYQFGMDDKNTA | ——— | 0.12 | ——— | Antigenic | Non-Allergen | 2.59E-14 | Non-Toxic |
| ——— | 89 | 104 | 16 | KEKIFMKFAPRMSLDA | ——— | 0.15 | ——— | | | 3.88E-12 | |
| ——— | 190 | 204 | 16 | YQGYIDYQFGMDDKNT | ——— | 0.24 | ——— | | | 3.70E-19 | |
| ——— | 244 | 259 | 16 | EGLAGKTTGFGHYLAV | ——— | 0.3 | ——— | | | 3.59E-13 | |
| ——— | 49 | 64 | 16 | GESSHDYLEMEFGGRS | ——— | 0.32 | ——— | | | 1.40E-05 | |

**Table 2**
OmpK vaccine construct (OVC) physiochemical properties and characteristics.

| Physiochemical Properties | | | | |
|---|---|---|---|---|
| **Parameters** | | **Results** | | |
| Total no. of Amino Acids | | 269 | | |
| Molecular weight | | 30206.85 | | |
| Theoretical isoelectric Point (pI) | | 5.64 | | |
| Negative Charged Residues (Asp+Glu) | | 31 | | |
| Positively Charged Residues (Arg+Lys) | | 23 | | |
| Formula | | $C_{1379}H_{2015}N_{347}O_{402}S_{10}$ | | |
| Total no. of Atoms | | 4153 | | |
| Instability Index | | 12.88 | | |
| Aliphatic Index | | 65.99 | | |
| Grand average of hydropathicity | | -0.377 | | |
| Solubility | | Insoluble | | |
| **Characteristics** | | | | |
| **Antigenicity** | | **Allergenicity** | **Toxicity** | |
| **Protective score** | **Probability** | **Probability** | **Toxicity Score** | **Probability** |
| 0.6302 | Antigenic | Non-Allergen | $5.36\times10^{-7}$ | Non-toxic |

### 3.7. Codon optimization and final transformation vector

GenSmart™ Codon Optimization webserver was used to perform codon optimization on the OVC construct in accordance with the plant codons to facilitate its expression. The GC content found in the OVC was 41.02% showing a significant amount of expression potential in the host system. The final transformation binary vector pEXP-OmpK (Fig. 3A) was constructed via Gateway® cloning for the transformation of tobacco. The pEXP-OmpK consisted of *OmpK* transgene from *V. anguillarum* with a 6×His-tag attached at the C-terminal. The pEXP-OmpK contained the *neomycin phosphotransferase* II (nptII) gene (kanamycin) for the selection of transformed bacterial colonies and for the selection of transformed plants *hygromycin phosphotransferase (hpt)* gene (hygromycin) was present. Fig. 3B shows PCR confirmation of correct integration and successful transformation of the *OmpK* gene in *Agrobacterium tumefaciens*.

### 3.8. Transgenic tobacco plant regeneration and confirmation of OmpK via PCR

The explants of tobacco after infection with *Agrobacterium tumefaciens* harboring pEXP-OmpK were placed on a co-cultivation medium for two days. After that, the explants were shifted to hygromycin (30 mg/L) supplemented RMOP medium. On the selection medium, the untransformed plants started bleaching after 10 days while in the case of transformed explants green shoots appeared on the selection medium (Fig. 4). The regenerating shoots were transferred to a fresh hygromycin (30 mg/L) supplemented RMOP medium in glass jars. Transformed tobacco plants were then acclimatized to the soil. The development of shoots showed that the plants were putatively transformed, which was further confirmed by PCR using different sets of primers. The expected bands were obtained in transgenic lines confirming the *OmpK* gene integration within the tobacco nuclear genome (Fig. 5). Wild-type plants (WT) showed no band.

### 3.9. Copy number determination via qRT-PCR

qRT-PCR was carried out on PCR-confirmed transgenic lines to determine the *OmpK* gene copy number. For the *OmpK* and *β*-actin gene (control), standard curves were obtained by using serial dilution with slopes values of −1.289 and −1.234, and correlation coefficients values of 0.995 and 0.943, respectively. From the standard curve the starting quantities of the *OmpK* gene ($SQ_{trans}$) and *β*-actin gene ($SQ_{end}$) were evaluated (Data not shown) and the transgene (*OmpK*) copy number was determined by using the following formula:

$$\delta rline = rline\left[(\delta SQ_{trans}/SQ_{trans})^2 + (\delta SQ_{end}/SQ_{end})^2\right]^{1/2}$$

A maximum of two copies of the *OmpK* gene were integrated into the OmpK transgenic lines of tobacco.

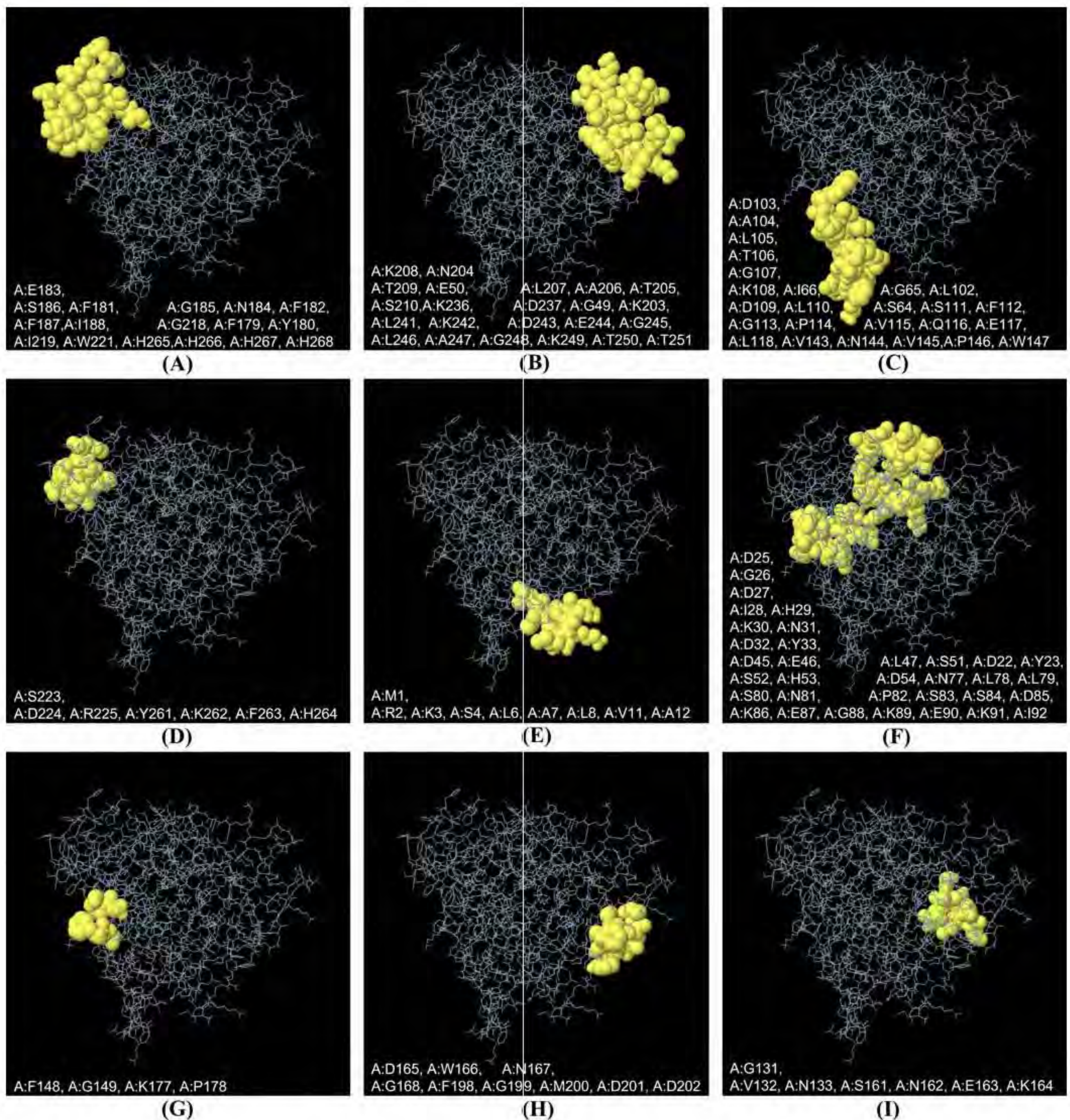


**Fig. 2.** Detection of discontinuous B cell epitopes on "Model 2" of OVC. (A-I) Discontinuous B cell epitopes (yellow), Model 2 (grey sticks), detected epitope name (white).

### *3.10. OmpK protein confirmation and quantification*

Western blot was done to confirm the transgenic OmpK protein expression. The molecular weight of OmpK protein along with His-tag was anticipated to be 30.21 kDa, corresponding to its monomeric form. A clear band of transgenic protein in monomeric form was visible in all independent transgenic lines of tobacco plants (Fig. 6). Protein extracted from six independently generated transgenic lines was loaded in corresponding lanes (lanes 1–6). The Fig. 5 also shows the Ponceau staining of the protein samples (Fig. 6A) and the molecular weight marker. No band was detected in the WT tobacco plants. Quantification of transgenic protein was done via ELISA by following Verma et al. (2008). The maximum expression of OmpK protein calculated was 0.38% of TSP in transgenic lines. The transgenic line expressing the protein in highest amount was used for further animal trials.

### *3.11. OmpK proves to be significantly immunogenic in mice and fish*

To investigate the humoral response, the mice were immunized orally and subcutaneously. The specific antibodies against OmpK in the serum from immunized mice and fish were assayed by indirect ELISA. Promising results were shown by OmpK expressing transgenic tobacco

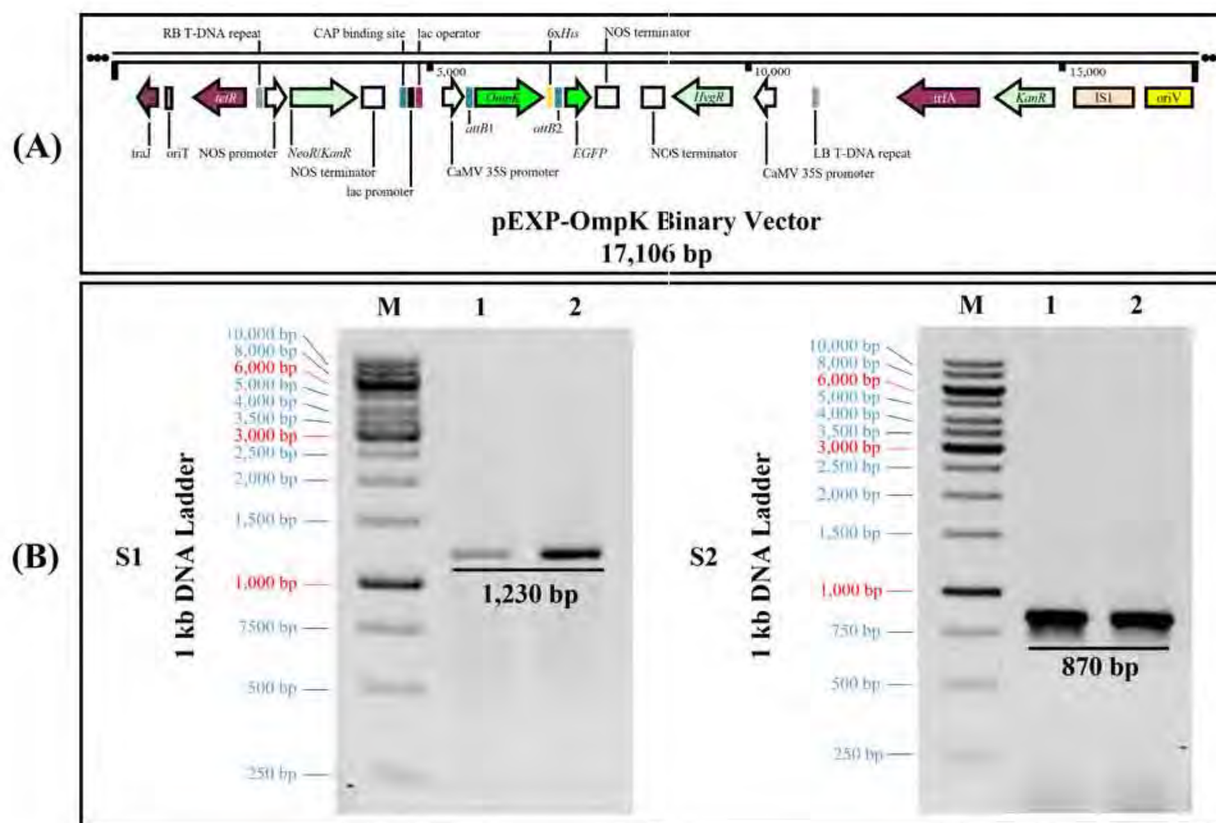


**Fig. 3.** (A) Schematic representation of pEXP-OmpK binary vector showing expression cassette along with pDEST-pGWB5 backbone. traJ: oriT-recognizing protein; oriT: incP origin of transfer; tetR: tetracycline resistance regulatory protein; RB T-DNA repeat: right border repeat from nopaline C58 T-DNA; NOS promotor: nopaline synthase promoter; *NeoR/KanR*: a gene conferring resistance to kanamycin; NOS terminator: nopaline synthase terminator; CAP binding site: *E. coli* catabolite activator protein; lac promotor: promoter for the *E. coli* lac operon; lac operator: lac repressor encoded by lacI; CaMV 35 S Promoter: strong constitutive promoter from cauliflower mosaic virus; attB1/B2: Gateway® recombination sites; *OmpK*: *OmpK* gene from *V. anguillarum*; 6xHis: 6xHis affinity tag; *EGFP*: the original enhanced GFP; NOS terminator: nopaline synthase terminator and poly(A) signal; *HygR*: a gene conferring resistance to hygromycin; LB T-DNA repeat: left border repeat from nopaline C58 T-DNA; trfA: trans-acting replication protein that binds to and activates oriV; IS1: insertion sequence 1; OriV: incP origin of replication. (B) Confirmation of *OmpK* gene integration in *Agrobacterium tumefaciens*. M: 1 kb DNA ladder; S1: PCR with primers set 1; S2: PCR with primers set 2; 1,2: DNA extracted from 2 transformed colonies.

plant when ELISA was carried out on collected blood sera. Considerably high IgG responses were shown in both mice groups F (oral delivery) and G (subcutaneous delivery) (Fig. 7). The antibody levels in the mice subcutaneously immunized were greater than those immunized via the oral route. The fish immunized orally with transgenic tobacco harboring OmpK also showed significant level of IgG as compared to its control groups (H&I). The values of IgG in fish were slightly lower than that of orally immunized mice group F (Fig. 7).

## 4. Discussion

The present study demonstrates the first report on the expression of OmpK vaccine antigen in higher plants against Vibriosis, potentially targeting multiple *Vibrio* species due to the conserved nature of OmpK in all *Vibrio* species. The vaccine construct was first evaluated for its immunogenic potential and other properties using bioinformatics tools. We chose tobacco for protein expression, due to its high biomass, scalability, and potential for high foreign protein expression. The OmpK was expressed via *Agrobacterium*-mediated transformation. The plant-derived vaccine showed significant immunogenicity in mice via oral and subcutaneous routes.

*Vibrio anguillarum* and other vibrio species, causing vibriosis, pose a significant risk for the aquaculture industry around the world (Xing et al., 2020). As an innovative technological approach, in silico designing of vaccines using bioinformatics tools and biological databases has allowed the accurate prediction of vaccine properties and the development of highly efficient and safe vaccines (Baliga et al., 2018). In the present study, we used a reverse vaccinology approach guided by immune informatics to investigate the potential of OmpK as a potential vaccine. A plant-based expression system is an attractive, cost-effective, scalable, and safe platform for protein expression. The transformed plants can be grown easily at a site where the product is needed, and it can be administered in the form of partially processed or unprocessed material (such as plant leaves or the whole plant) orally, subcutaneously, or by intra-peritoneal means (Saba et al., 2020; Waheed et al., 2011). Most vaccines available in the market are quite costly since these are made using expensive fermenter-based production facilities. The price of a cooling chain and its maintenance, as well as the cost of injection delivery, can be substantial. Vaccines against diseases must be produced efficiently so that they can be made widely available at a low price. In this regard, prophylactic vaccine design followed by in silico analysis of the *V. anguillarum* target gene *OmpK* against vibriosis is promising, as it can not only save researchers from costly trial-and-error experimental failures but also reduce the costs associated with vaccine design by providing a clear path forward (Ji et al., 2020; Xu et al., 2019a). Further, for aquaculture, fish vaccines are required on a large scale and this problem can be solved by plant-based production platforms.

To control many diseases of aquaculture, vaccination is an effective strategy. In many parts of the world, for a variety of aquatic species, *V. anguillarum* has been identified as a major causative agent. Few vaccine formulations have been proven to be protective against vibrio infections,

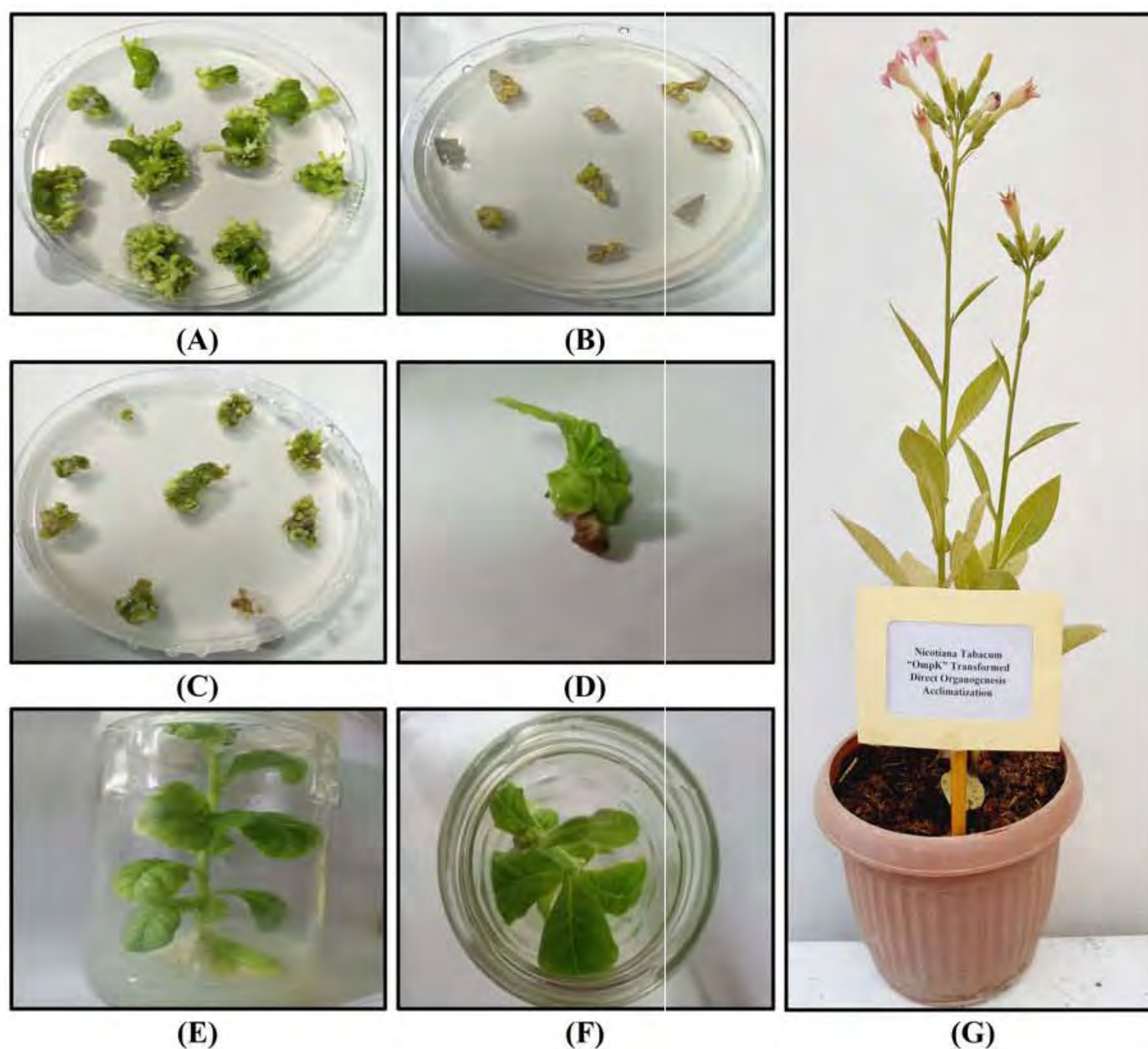


**Fig. 4.** OmpK transformed tobacco regeneration. (A) WT tobacco explants on an RMOP medium. (B) Untransformed explant on selection medium. (C) OmpK transformed plant on selection media. (D) Shooting of transformed explants on selection media. (E & F) Transgenic plant rooting. (G) Acclimatization of the transgenic plant.

such as bacteria killed by formalin or inactivated by heat (Hamod et al., 2012; Newman, 1993). However, whole cell-based vaccines (WCV) may cause adverse effects in vaccine recipients on parenteral administration. Whereas oral administration of WCV frequently requires high doses and produces short-term immunity. Additionally, WCVs have a poor capacity to elicit cellular immune responses (Pace et al., 1998; Qin et al., 2022). Thus, an effective and safe vaccine needs to be developed to control vibriosis.

Outer membrane proteins (OMPs) are highly immunogenic due to their exposed epitopes on the surface and thus are promising potent vaccine candidates. Host defense systems can easily identify fractions of the OMPs as foreign antigens and play an important role in eliciting the host immune response (Frans et al., 2011; Hamod et al., 2012; Hong et al., 2009; Khushiramani et al., 2012; Wang et al., 2003). Qian et al. (2008) have reported the Outer membrane protein K (OmpK) as a protective antigen against fish vibriosis. OmpK exposure to the external environment is an important characteristic that makes it an effective candidate for the development of a broad-spectrum vaccine (De Groot et al., 2002; Ellis and Kuehn, 2010; Zhang et al., 2008). OmpK has been reported to have the ability to elevate the level of antigen-specific antibodies, innate immune response (IL-1$\beta$, TLR5M, and IL-12p40), cytokine production (IFN-γ and T-bet), and T cell numbers (CD4–1, CD4–2, and CD8α) in vaccinated fish (Hamod et al., 2012; Lee et al., 2021; Mao et al., 2007). Vaccines should be safe, effective, and able to provide long-term protection against bacterial diseases (Li et al., 2010). Without efficient vaccination, controlling the spread of vibriosis is difficult. Furthermore, a vaccine that can effectively curb the existing situation of vibriosis has not yet been available in the market (Ji et al., 2020; Xu et al., 2019b).

The immune response would be robust when all possible CTL, HTL, and linear B cell epitopes are identified (Chen et al., 2021). In our study, a total of 269 amino acid residues were used in the production of the OVC. Various vaccination characteristics were analyzed by processing the full-length OmpK (along with 6x Histidine residues) sequence as a vaccine. In silico physiochemical analysis of the vaccine determined that the OVC was very stable showing high stability index, but insoluble following over-expression. The OVC also exhibited high antigenic, non-allergenic, and non-toxic properties. The PI value indicated the vaccine was acidic in its composition. Based on its expected physicochemical properties and high scores across all criteria, this OVC appeared a potent vaccine to fight vibriosis. The 3-D structure was predicted by 3-D structural modeling of the OVC. ERRAT quality factor, Ramachandran plot, and Z-score predicted the well-performance of OVC. Using three-dimensional models, researchers can foresee the presence of discontinuous B cell epitopes, which are necessary for vaccine-induced immune system activation. The online server projected many discontinuous B cell epitopes, pointing to the promising potential of the designed OVC for immunological activation.

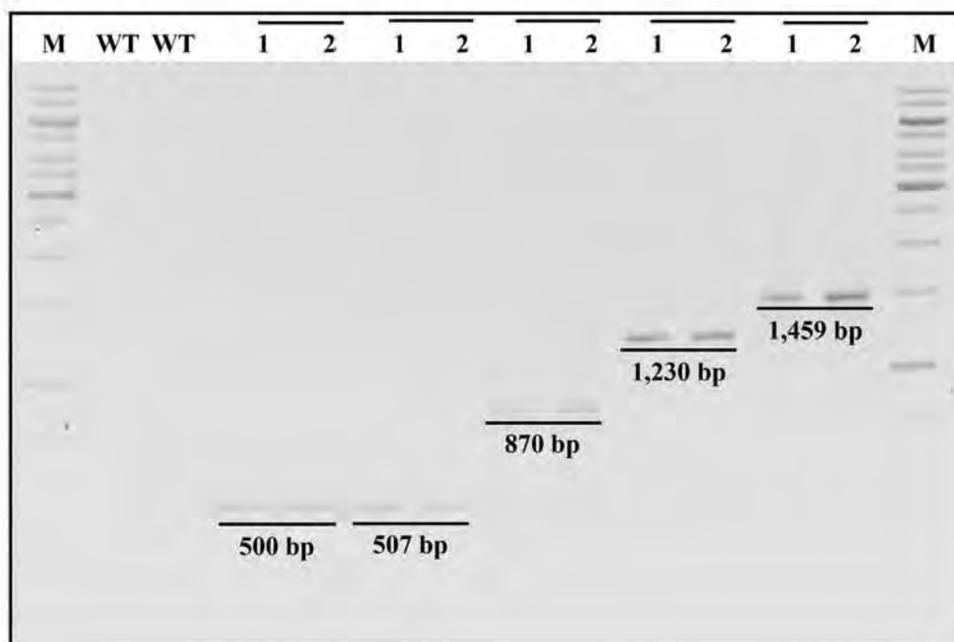


**Fig. 5.** *OmpK* gene integration confirmation in transgenic tobacco. M: 1 kb DNA ladder; 1, 2: two OmpK independent transgenic lines; WT: wild-type plant; The bar line with alphabets A-E: a different set of primers.

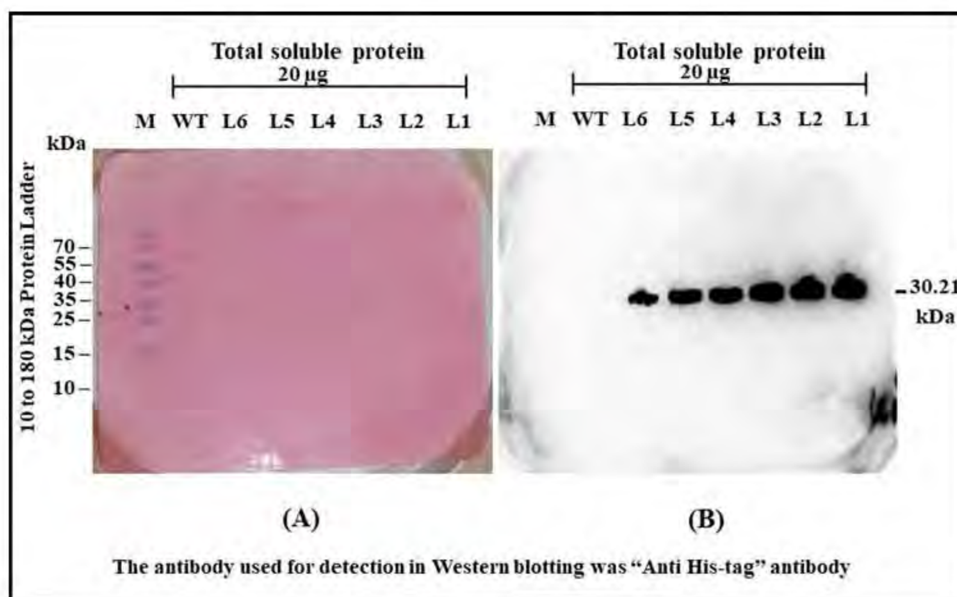


**Fig. 6.** Detection of OmpK protein extracted from transformed tobacco plants (A) Ponceau staining (B) Western blotting of protein extracted from transformed and wild type tobacco plants. 20 μg of total soluble fraction of protein extracted from tobacco leaves was loaded in each lane. L1-6: six independently generated OmpK transgenic tobacco lines; WT: wild-type plant; M: 10–180 kDa molecular weight marker; anti-His antibody was used for detection.

In both the invertebrate and vertebrate lineages, a crucial role is played by Toll-like receptors (TLRs) in their defense against invading pathogens. Activation of subsequent signaling pathways occurs upon recognition of ligands by respective TLRs (Akira and Takeda, 2004; Medzhitov, 2001; Purcell et al., 2006). Studies have shown that the regulation of TLR-5 paralogs in fish is differentially regulated in response to bacterial infection and ligand stimulation in the tissues (Hwang et al., 2010). Soluble TLR-5 participate in host defense against microbes in human as well as in fish (Tsujita et al., 2006). Thus, our study examined the interaction of the suggested vaccination construct with the TLR-5 by performing docking analysis using a webserver ClusPro 2.0. The docking analysis indicated sufficient and meaningful contact between the vaccine design and the immune cell receptor. We also carried out the expression analysis of OVC. The sequence of the planned vaccine construct was optimized for the codon of the expression system into which it was to be introduced for high-level expression before it can be used in in vitro and in vivo experiments (Atapour et al., 2021). The vaccine sequence was optimized for the eukaryotic plant's codons. The total GC percentage of the OVC was 41.02%. This astoundingly high GC content value was potentially indicative of high protein expression in the host.

For the aquaculture industry, there is a need for oral vaccines as a convenient and ideal mode of vaccination, so that the fish can be protected from stress that it encounters through other methods of

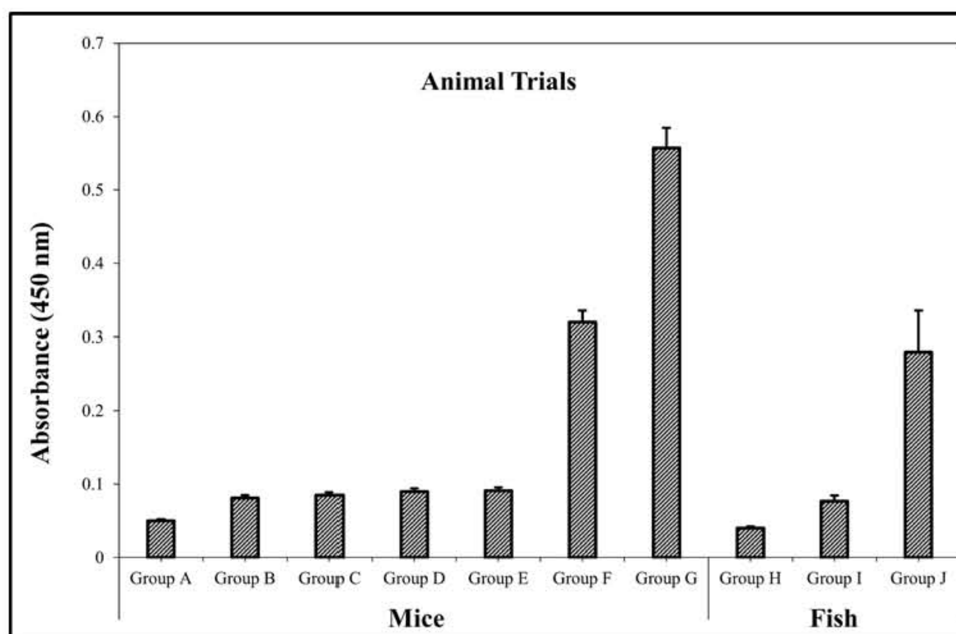


**Fig. 7.** Antibodies level in the blood sera of OmpK immunized animal models. Mice groups (A-G) Group A: Normal mice (No dose); Group B: PBS solution oral delivery (control); Group C: PBS solution subcutaneous delivery; Group D: WT tobacco TSP oral delivery; Group E: WT tobacco TSP subcutaneous delivery; Group F: Transgenic tobacco TSP oral delivery; Group G: Transgenic tobacco TSP subcutaneous delivery. Fish groups (H-J) Group H: Normal group on commercial feed; Group I: WT tobacco oral delivery mixed in feed pellet; Group J: Transgenic tobacco oral delivery mixed in feed pellet.

vaccination. Studies have shown that immunity in fish can be achieved via oral routes (Embregts and Forlenza, 2016). Oral vaccines can induce immunity at both the mucosal level and systemic immune level. Oral vaccines can be a better option to counter the pathogens that infect the organisms via mucosal routes such as *Vibrio* spp., as compared to injectable vaccines (Lee et al., 2021). For the manufacturing of immunogenic proteins as oral vaccines, plant-based production platforms are well-established and promising biotechnology-based bioreactors (Ma et al., 2013).

Few studies report the expression of OmpK in prokaryotic expression systems. The antigens OmpK and LamB have been recently expressed in microalgae (*Nannochloropsis*. sp.) and duckweed, respectively, against vibriosis as vaccine candidates (Abidin et al., 2021; Heenatigala et al., 2020). However, to date, there has been no study on the expression of OmpK as a vaccine antigen against vibriosis in higher plants such as tobacco. In this study, we transformed tobacco via *Agrobacterium*-mediated transformation to stably express the OmpK antigen. The stable expression and inheritance of the *OmpK* gene were also verified in the next generation (T1 obtained via seeds of T0 generation, data not shown). Confirmation of transgene in the nuclear genome of tobacco was carried out by PCR, whereas, to determine the copy number of transgene in transgenic tobacco qRT-PCR was performed. The maximum integrated copy number was found to be two. The number of copies of the integrated transgene varies when carried out by *Agrobacterium*-mediated transformation as it randomly integrates the transgene into the plant's nuclear genome. However, the average number of copies inserted into the genome is usually 1–2 (Wen et al., 2012).

Tobacco was chosen for the OmpK protein expression because of its high totipotency, high biomass, scalability, eukaryotic post-translational modifications, and well-established tissue culturing and transformation protocols (Jube and Borthakur, 2007; Lössl and Waheed, 2011). For the development of oral vaccines, an adequate amount of transgenic protein is required to meet the optimum required dosage (Saba et al., 2020). In the current study, the maximum expression level of OmpK protein was about 0.38% of total soluble protein (TSP) in our transgenic lines L5 and L6. We confirmed the expression of OmpK in the leaves of transgenic tobacco via Western blot. The OmpK band equivalent to 30.21 kDa (monomeric form) was observed in transgenic lines.

Immunization by feeding the plant biomass is of particular interest as it is easy to use and less costly. There have been studies in which the immunogenic potential of plant-expressed vaccine antigens was tested by feeding transgenic tobacco plant material to mice and fish (Cho et al., 2018; Ruhlman et al., 2007; Saba et al., 2020). In the current study, the immunogenic potential of transgenic tobacco expressing the OmpK antigen was investigated in mice and fish. Mice were orally and subcutaneously immunized by giving the OmpK protein. The results showed that oral delivery and subcutaneous delivery of OmpK induced the humoral response in mice. In fish, oral immunization resulted in significant immunogenic response. Few previous studies on OmpK as an antigen produced via the prokaryotic expression system also showed immunogenicity in animal models (Li et al., 2010, 2008; Zhang et al., 2008). For oral delivery, one of the main hurdles is the denaturation and degradation of antigens by the stomach's strong acidic environment (Embregts and Forlenza, 2016; Saba et al., 2020). However, in plant-based expression, the encapsulation provided by plant cells could help the antigens bypass the harsh acidic environment of the digestive tract and can elicit the humoral response via the gut epithelium (Kwon and Daniell, 2015).

The present study reports the successful expression of OmpK antigen in tobacco plants via stable nuclear expression. Significant immune response in mice and fish was achieved by plant-expressed protein. The current research is a significant scientific contribution to the limited existing vaccine research against fish diseases and has a strong potential to develop a vaccine against vibriosis for use in aquaculture.

## Ethics approval

For animal trials, ethical approval was obtained from the Bioethics committee of the institute.


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## CRediT authorship contribution statement

**Khalid Fatima:** Formal analysis, Methodology. **Waheed Mohammad Tahir:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Sameeullah Muhammad:** Project administration, Investigation, Formal analysis. **Elahi Iqra:** Visualization, Investigation, Data curation. **Malik Muhammad Suleman:** Writing – original draft, Methodology, Investigation, Formal analysis. **Saba Kiran:** Validation, Investigation, Formal analysis. **Gurel Ekrem:** Resources, Methodology, Data curation. **Batool Neelam:** Visualization, Investigation. **Ijaz Fatima:** Methodology, Investigation, Formal analysis.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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