Chapter 1
Plant Molecular Farming: Concept and Strategies

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Abstract Plant molecular farming refers to a plant-based approach to produce recombinant proteins and secondary metabolites. The utilization of plants as a bioreactor for the production of valuable recombinant proteins has shown some unique advantages in comparison to other expression systems. With the high cost and increase in demand for therapeutics and industrial proteins, plant molecular farming has gained interests over the years for its promising low-cost productions at large scales. This chapter starts off with an introduction to plant molecular farming and discussions on its recent updates. It is then followed by describing the principle of the approaches, which explains the processes involved in plant molecular farming, different plant species that are utilized as expression hosts for the production of recombinant proteins and secondary metabolites, and types of recombinant proteins produced. This chapter also discusses various transformation strategies that are utilized as well as the advantages and challenges of plant molecular farming.

Keywords Edible vaccine · Genetic engineering · Nuclear transformation · Plastid transformation · Recombinant proteins

Abbreviations

2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
2iP(IPA)	N^6-(2-isopentenyl)adenine
BAP	6-Benzylaminopurine
CAMV-35S	Cauliflower mosaic virus 35S

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COVID-19	Coronavirus disease-19
ELP	Elastin-like polypeptide
FDA	Food and Drug Administration
GMP	Good manufacturing practice
GR2	Second-generation Golden Rice
HFBI	Hydrophobin I
hGH	Human growth hormone
HPV	Human papillomavirus
IAA	Indole-3-acetic acid
mAb	Monoclonal antibody
NAA	1-Naphthaleneacetic acid
NHL	Non-Hodgkin's lymphoma
PEG	Polyethylene glycol
PHAs	Polyhydroxyalkanoates
PMF	Plant molecular farming
PVX	Potato virus X
scFv	Single-chain variable fragment
T-DNA	Transfer DNA
TMV	Tobacco mosaic viruses
USDA	United States Department of Agriculture
VLP	Virus like particles

1.1 Introduction

The concept of "farming" is no longer restricted to the conventional perception of just the process of rearing animals or planting crops for food. Advancements in biotechnological fields such as molecular biology and genetic engineering have paved the way for molecular farming, which allows for a whole new spectrum of resources that can be harnessed for a variety of potential applications. Efforts in developing targeted gene editing tools in plants have laid the groundwork for plant molecular farming (PMF), to efficiently and precisely produce organisms that express specific recombinant proteins and secondary metabolites as desired (Mao et al. 2019). The concept of PMF differs slightly in other approaches of plant genetic engineering. Plants can be genetically engineered to confer specific desired qualities or characteristics to the plant, typically seen in agriculture, wherein to increase the yield or durability of crops, the resultant recombinant protein is induced into the plant to increase the production of a desired metabolite (Fischer and Schillberg 2016). Rather than adding value to the plant, PMF aims to specifically produce the desired products via a commercialized, large-scale approach (Fischer and Schillberg 2016). Achieving this feat would allow for inexpensive access to much-needed medicines and pharmaceuticals to virtually everyone globally.

The use of plants in molecular farming has now seen almost 40 years of development since the first plant-produced pharmaceutical protein (human growth hormone) from tobacco and sunflower calluses in 1986 (Barta et al. 1986) and the first

plant-produced antibody in transgenic plants in 1989 (Hiatt et al. 1989). It was only 6 years later that the first oral immunization was observed on mice after consuming transgenic potatoes, making it the first edible plant-based vaccine to be produced (Haq et al. 1995). However, it took more than 20 years for a plant-based vaccine to gain the world's first regulatory approval when a Newcastle's disease vaccine for poultry produced in transgenic tobacco cell lines was approved by the United States Department of Agriculture (USDA) in 2006 (Sparrow et al. 2007). Unfortunately, the vaccine was never commercialized and brought to the market by the developing company (Thomas et al. 2011). It was also in the same year that the production of a monoclonal antibody (CB-Hep.1) used to produce a vaccine for hepatitis B was approved by Cuba's Medication Quality Control Agency (Sparrow et al. 2007), marking the first approval granted for the commercial use of plants in molecular farming. Further success in human clinical trials was observed as a plant-based vaccine produced in tobacco was effective in eliciting an immune response against follicular lymphoma (McCormick et al. 2008; Thomas et al. 2011).

Despite the considerably long developmental history of plant-based biopharmaceutics, the commercialization of such products in the market is still at its infancy. So far, there has only been one such product that was authorized to enter the market after its completion of phase I–III clinical trials (Schillberg and Finnern 2021; Zimran et al. 2018). Another notably historic use of a PMF product was during the not-so-recent Ebola outbreak, where ZMapp, a cocktail of monoclonal antibodies produced in transgenic tobacco (*N. benthamiana*), was authorized for emergency use in humans despite lacking the necessary human clinical trials at the time (Fanunza et al. 2018; Hiatt et al. 2015; Qiu et al. 2014). While ZMapp showed great promise during the initial use, further clinical trials conducted after the outbreak remained inconclusive (The PREVAIL II Writing Group for the Multi-National PREVAIL II Study Team 2016). Fortunately, the efforts in PMF development were not discouraged, as the call for action for mass-produced biopharmaceuticals arrived with the onset of the coronavirus disease-19 (COVID-19) pandemic. The urgency for preventative measures and treatments for COVID-19 were the focus of the efforts of researchers globally, including biopharmaceutical manufacturers such as Medicago, Kentucky Bioprocessing, iBio, and Newcotiana (Kumar et al. 2021). This highlights the potential reliance on a stable, efficient, and high-throughput method for the production of pharmaceuticals and recombinant proteins alike in the future, a niche that is perfect for the expansion of PMF (see also Chap. 12).

In comparison to other expression systems such as bacteria, yeast, mammalian cells, or insect cells, plants have been the popular platform used in molecular farming as it offers significant benefits over the other expression systems (Diamos et al. 2020; Schillberg and Finnern 2021; Shanmugaraj et al. 2020). The robustness, scalability cost, and relative safety of plant recombinant proteins (which shall be discussed in further sections) certainly boost the potential of plants in molecular farming and are the reasons why plants are the general preferred option in molecular farming. This chapter serves as an introduction to the various concepts within PMF as well as the challenges faced in its implementation. Further, the diverse approaches and optimization strategies undertaken to further develop PMF's viability shall also be discussed.

1.2 Concept

1.2.1 Plant as Expression System for Recombinant Protein Production

Utilization of transgenic plants for the production of various recombinant proteins and secondary metabolites offers an appealing alternative to other commonly used expression systems. In general, the process of PMF involves the development of transgenic plants as bioreactors through recombinant DNA technology. This entire process begins with the selection of a gene of interest. This candidate gene encodes for proteins that are able to express therapeutic properties such as vaccines and antibodies as well as industrial proteins such as enzymes or even diagnostic reagents. Following the selection of the gene of interest, the gene is inserted into a vector. The key to successful PMF is increased expression of recombinant proteins and secondary metabolites. Hence, expression cassettes play an essential role in ensuring an improved level of recombinant protein production (Makhzoum et al. 2014). Generally, these plant expression vectors consist of several components including the origin of replication, multiple cloning sites, and plant selectable markers (Low et al. 2018). The origin of replication site is an A + T-rich region where the replication process is initiated by unwinding the DNA (Low et al. 2018; Rajewska et al. 2012). The multiple cloning site has multiple restriction sites that can be cut by specific restriction enzymes, which permits the insertion of gene of interest allowing easy cloning of the gene (Crook et al. 2011). The plant selectable markers on the other hand play an essential role in providing proof of successful insertion of gene into vector. In terms of plant transformations, commonly employed vectors include the *Agrobacterium tumefaciens*-based Ti plasmid vector and plant RNA virus vectors to produce transient transformations allowing the transgene DNA to be expressed in the host, but it is not integrated into the host's germline DNA (Clark and Maselko 2020).

The vector containing the gene of interest is then introduced into plant cells. Prior to the introduction, a suitable plant species and its tissue need to be selected. This step is crucial as the physiological, morphological, biochemical, and genetic properties of each plant species may influence its success in terms of biomass and recombinant protein yield, capability in posttranslational modifications, and determination of polypeptide structural stabilities (Leite et al. 2019). Utilizing plant species with watery tissues such as tomato would also lead to a lower cost of downstream processing due to an easier process of extracting recombinant proteins compared to plants with dry tissues (Obembe et al. 2011). Once a suitable plant species has been selected, the transgene will be integrated into the plant cells by either stable or transient transformation process. A stable transformation refers to the integration of the gene of interest into the host cell genome, which results in the production of transgenic lines (Stepanenko and Heng 2017). This stable transformation can be done by nuclear transformation or plastid transformation. In transient transformation on the other hand, the gene of interest is present in an extrachromosomal manner, and it

does not affect the host genome stability (Honda et al. 2019; Ji et al. 2014). This transient transformation can be done by agroinfiltration, viral infection, or magnification system, which will be discussed further in the following sections. Successful transformants are identified and selected to be regenerated into transgenic plants.

The regeneration of transgenic plants is carried out by culturing successful transformants in plant growth regulators. Two of the major plant growth regulators utilized in plant tissue culture are cytokinins and auxins (Abiri et al. 2016). Auxins play an essential role in the formation of an unorganized and undifferentiated mass of cells known as callus (Rahayu et al. 2016). Commonly employed auxins include 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), picloram, dicamba, and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Abiri et al. 2016). Cytokinins on the other hand are essential to promote cell division and cell expansion in the development of leaf cells (Wu et al. 2021). Commonly used cytokinins include zeatin, kinetin, 6-benzylaminopurine (BAP), thidiazuron, and N^6-(2-isopentenyl)adenine (2iP(IPA)) (Bhatia 2015). An increased auxin-to-cytokinin ratio promotes the regeneration of roots, while an increased cytokinin-to-auxin ratio promotes the regeneration of shoots (Ikeuchi et al. 2013). This method of inserting a gene of interest into plant cells or tissue from explants and regenerating transgenic plants is utilized in stable transformation strategies. Alternatively, in transient transformation strategies, the gene of interest in vectors such as *Agrobacterium tumefaciens* and plant viruses are directly inserted into the intracellular space of the plant leaves by vacuum infiltration, syringe infiltration, or viral injection (Floss and Conrad 2013; Shanmugaraj et al. 2021). As a result, the plants will produce the desired recombinant proteins. Subsequently, the harvest will undergo downstream processing to extract and purify the desired recombinant proteins. The processes involved in plant molecular farming are summarized in Fig. 1.1.

1.2.2 Plant as a Bioreactor for Production of Recombinant Proteins and Secondary Metabolites

For a long time, breakthroughs in plant biotechnology were directed at the significant enhancement and transformation of specific plant components such as carbohydrates, proteins, lipids, and vitamins. This enhancement is referred to as "transgenic plants" where the plants can produce a broad range of foreign genes and enable massive protein and metabolite synthesis in pharmaceutical and agricultural environments (Franken et al. 1997; Sharma and Sharma 2009). The need for transgenic plants as bioreactors, on the other hand, is a comparatively modern approach that is rapidly developing. It comprises genetic altering of the host plant by introducing and expressing the gene of interest. Industrial enzymes, pharmaceutical proteins, secondary metabolites, bioactive peptides, vaccine antigens, and antibodies are some of the often-produced proteins and metabolites in plant bioreactors (Lau and Sun 2009; Sharma and Sharma 2009). For instance, during this SARS-CoV-2

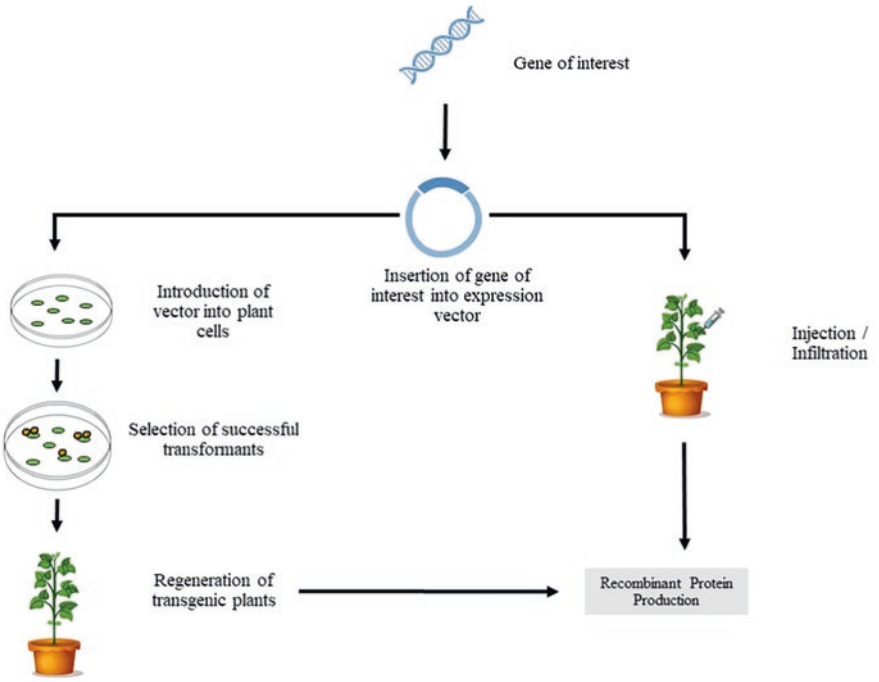

Fig. 1.1 Plant molecular farming process

pandemic era, the plant-based vaccine has obtained more attention for its cost-effective and easy manufacturing process. Researchers were racing to produce an efficient vaccine against the virus using plants as bioreactors (Kumar et al. 2021). One such company, Medicago, worked on using a close relative of the tobacco plant. They utilized the viruslike particle (VLP) technology and grew within the plant species to produce the vaccine candidate against the pathogen. Medicago also has successfully developed a VLP-based influenza vaccine using the Proficia plant as a bioreactor (Kumar et al. 2021). This indicates the success of using plants as bioreactors to produce vaccine proteins or candidates. Pereira et al. have successfully demonstrated that plant bioreactors are exhibiting promising results for enzyme production. They worked with the *lipA* gene originating from *Acremonium alcalophilum* that encodes for a lipase, which has multiple industrial uses such as in the field of biofuels and food processing industries (Pereira et al. 2013). The *lipA* gene has successfully been expressed in the tobacco plant, and the plant-produced recombinant LipA enzyme is highly active. This indicates that the plant bioreactor also exhibits promising outcomes in producing industrial enzymes, and the tobacco plant is a suitable bioreactor for fungal enzyme production (Pereira et al. 2013; see also chapter "Plant Molecular Farming for the Bulk Production of Industrial Enzymes").

However, the expression hosts of the recombinant proteins and secondary metabolites are not limited to tobacco plant. Researchers are discovering and studying multiple potential plant expression hosts that can be utilized as a bioreactor. They have identified and established some other plant bioreactors as the expression host, which will be discussed in the following sections (see also chapter "Molecular Farming of Pharmaceutical Proteins in Different Crop Systems: A Way Forward").

1.2.2.1 Tobacco

Tobacco is a widely used plant as a bioreactor in the field of plant biotechnology and is known as a "white mouse." It is the most preferred plant system for genetic modification and has been the major system for recombinant protein and secondary metabolites for the past 30 years. Tobacco is identified as one of the best plant bioreactors for its large biomass production and massive soluble protein production compared to any other plant bioreactors (Tremblay et al. 2010). The advantages of using tobacco as bioreactors include high yield; ability to produce a wide range of therapeutic, industrial, and agricultural proteins; and a short time for large-scale production (Gutiérrez et al. 2013; Tremblay et al. 2010). One considerable downside of using tobacco as a bioreactor is the shelf life of the plant. The transgenic tobacco plant should have shorter half time after reaching maturity and needs urgent processing of the protein (Sharma and Sharma 2009). Despite the advantages of producing a high yield, there are limitations, whereby certain recombinant protein yields will be significantly low. To overcome this, the transgenic tobacco plant could be designed together with other proteins or fusion tags to increase the yield. To demonstrate this, a study conducted by Gutierrez et al. has fused elastin-like polypeptide (ELP), hydrophobin I (HFBI), and Zera protein to increase the desired recombinant protein level. In their study, they tested the protein body formation in *N. tabacum* plant (Gutiérrez et al. 2013). These tags have shown promising results where the protein body level is significantly high and suggest that these two tags could be a potential fusion partner in tobacco plants to increase the yield of the recombinant protein (Gutiérrez et al. 2013). As mentioned earlier, tobacco transgenic plants are used widely in multiple fields; for instance, they can also be used to produce recombinant silk protein. Spider silk is one of the powerful natural fibers and has multiple uses in the field of medicine including facilitating healing and connecting the wounded or cut skin (Kirsh 2017). However, the availability of the protein is limited and requires an alternative way to produce it in large amounts. Scheller et al. have demonstrated that the silk protein could be produced using the tobacco plant as a bioreactor. They have transformed many recombinant proteins from *Nephila clavipes*, which are responsible for silk production. They have shown promising data, where the silk protein observed in the plant tissues was approximately 100 kDa, and when produced in the plant, the protein showed strong heat stability (Scheller et al. 2001). These data show that tobacco is a good candidate for silk protein production and can be deduced that a foreign gene can be expressed in

the plant easily without many complications (see also chapter "Tobacco Plants as a Versatile Host for the Expression of Glycoproteins").

1.2.2.2 Rice

Rice is another available plant bioreactor option to deliver desired protein or metabolites to humans in the form of edible transgenic rice. It is the first-ever food crop to ever completely sequence and the genome available for the public view (McCouch and Herdt 2004). One of the well-known achievements of plant biotechnology and usage of rice bioreactors is the development of "Golden Rice." Vitamin A deficiency is a major nutritional condition that can be observed in children around the globe, especially in underdeveloped countries. To overcome this, a food-based vitamin A supplement was a sustainable option for the deficiency (Tang et al. 2009). Therefore, researchers worked on developing the Golden Rice, which is rich in beta-carotene, which in turn will be converted into vitamin A by the human body upon consumption of the rice. In 2004, the researchers Ingo Potrykus and Peter Beyer who developed Golden Rice performed their first field trial, which has shown promising results with the beta-carotene level being higher compared to the other grains. The first-generation Golden Rice had approximately 6 μg beta-carotene, which was lower than the recommended intake, but this was overcome in the same year with the second-generation Golden Rice (GR2), with approximately 35 μg beta-carotene (Tang et al. 2009; The Golden Rice Story n.d.). Tang et al. have confirmed that beta-carotene obtained from the Golden Rice is successfully converted into vitamin A upon consumption. They have tested the blood samples of healthy volunteers after consumption of Golden Rice serving and observed the retinol (vitamin A) level in the blood. The results showed a promising and significant result with 0.24–0.94 mg retinol after consumption indicating that the conversion of the beta-carotene was successful (Tang et al. 2009). Some of the benefits of using rice as a bioreactor include the following: (a) rice seed offers higher biosafety than other plant species, (b) rice endosperm has higher potential to efficiently express the recombinant protein of interest, and (c) it has the ability to express complex and functional proteins (Ou et al. 2014; Tang et al. 2009). Some of the limitation involved in using transgenic rice as a bioreactor is the public concern, the time required, and the attention provided to grow the crops. The crops require proper care and time to be fully developed and are sensitive to environmental factors such as pests and predator issues if grown in the field (McCouch and Herdt 2004). Besides the development of Golden Rice, rice could be used for other purposes as well. For instance, Yang et al. have used transgenic rice as a bioreactor to produce lipase B originated from *Candida antarctica*. They have successfully demonstrated the expression of lipase B in the rice seeds. The rice was transformed with the gene of interest, and a significant amount of lipase B has been expressed in the seeds. The transformed rice recorded the highest lipolytic activity with 85 units/g of dry seeds (Yang et al. 2014). The functions and effectiveness of the enzyme are identical to those used in industrial applications, which suggests that rice seed could be also used to produce

industrial enzymes besides using as a food-based supplement (see also chapter "The Use of Rice Seed as Bioreactor").

1.2.2.3 Maize/Corn

Maize or also known as corn is a highly produced crop across the globe and is majorly used for multiple purposes such as for human food, animal feed, and raw material for industrial use. Over the years, maize has been used in multiple transformation studies to encounter the high demand for maize grains for various purposes (Yadava et al. 2017). Some of the benefits of using maize as a bioreactor compared to other plants are the size of the seed, which allows the desired recombinant protein to accumulate in high concentration, which is beneficial in downstream processing. There will be low interference of other substances in the expression of the recombinant protein. The limitation of maize plants would be low yield despite the high concentration compared to other plant systems such as tobacco (Ramessar et al. 2008). Various studies have been performed on maize to achieve various goals; for instance, Barros et al. have succeeded in producing human monoclonal antibody 2G12 which is proposed to use against HIV infection. In this study, transgenic maize containing the 2G12 was bred together with South African maize lines, which resulted in a hybrid. The outcome was promising, where the hybrid transgenic maize expressed a higher level of antibody compared to the non-hybrid transgenic line (Barros and Nelson 2010). This shows another alternate option of increasing the recombinant protein expression in transgenic maize. This discovery could lead to large-scale production of neutralizing antibodies against HIV and could revolutionize the pharmaceutics industry (Barros and Nelson 2010). This finding also indicates the effectiveness and yield of the recombinant protein and secondary metabolite production. Table 1.1 lists multiple plant expression hosts that are used as plant bioreactors together with their advantages and disadvantages.

Table 1.1 Plant expression host with their advantages and disadvantages

Plant expression host	Advantages	Disadvantages	References
Tobacco	High-yield biomass, cost effective, time efficient	Half shelf life, extraction time point, low protein stability	Gutiérrez et al. (2013), Ramessar et al. (2008), Sharma and Sharma (2009), Tremblay et al. (2010)
Rice	Higher biosafety, efficient expression, expression of complex protein	Time consuming, public opinion	McCouch and Herdt (2004), Ou et al. (2014), Tang et al. (2009)
Maize	High concentration, low interference	Lower yield	Ramessar et al. (2008)

1.2.3 Types of Recombinant Proteins Produced by PMF

With the diverse options of plant systems available and being studied to produce recombinant proteins, there are a lot of successful plant systems that exist for a wide range of products, with a large influx of commercially viable products (Xu et al. 2018). The aim of developing a plant system as bioreactors is to produce a broad range of beneficial recombinant proteins. The type of proteins that are studied or successfully produced using a plant system includes edible vaccines, industrial proteins, diagnostic reagents, biopolymers, and therapeutic proteins, which will be discussed in the following sections.

1.2.3.1 Edible Vaccines

Vaccines are essential biological proteins that have been developed to provide protection and enhance the immune system's function against pathogens and disease. The vaccines could provide long-term or short-term protection based on the system used to develop them (Kurup and Thomas 2020). Conventional vaccines involve various tedious downstream processes, such as propagation, isolation, purification, and isolation of vaccine proteins. Another limitation includes that the conventional vaccine requires adjuvant as it needs to be administered through parenteral route and requires specific storage conditions such as the vaccine needs to be placed in cold temperature (Kurup and Thomas 2020). To overcome these issues, the concept of an edible vaccine has been established. The idea of edible vaccine refers to the usage of genetically modified plant or transgenic plants to carry and produce vaccine subunits against specific pathogens or diseases. The brief concept is that the vaccine proteins are introduced to the body via the intake of transgenic plants orally that are modified to produce vaccine proteins (Saxena and Rawat 2014). The advantages of edible vaccines include avoiding the need of using the invasive approach to administer vaccine where the intake is performed orally and avoiding the use of inactivated virus or toxins in the production of the vaccine protein (Saxena and Rawat 2014). Up to now, multiple plants have been studied and used as potential candidates to produce vaccine proteins, which include tobacco, potato, rice, maize, tomato, lettuce, spinach, and banana (Gunasekaran and Gothandam 2020; Saxena and Rawat 2014). For instance, an edible vaccine against hepatitis B has been produced using potatoes. Arntzen and his team from Arizona State University in Tempe have developed an edible vaccine that produces hepatitis B virus antigen, which upon intake will initiate the human immune system response that behaves as the booster shot against the virus. The development could replace the need for the conventional vaccine as the booster shot for hepatitis B (Khamsi 2005). Another discovery, wherein scientists from the University of Tokyo and Chiba University have entered the first human trial of edible cholera vaccine (MucoRice-CTB) using powdered rice, has shown promising results. The vaccine is developed by using Japanese short-grain rice plants, where it is genetically modified with an insertion of a gene

that produces a region of cholera toxin B, which is nontoxic to humans. The portion was selected to ensure that the protein can be identified by the human immune system and initiate a response. The vaccine is proved to be stable at room temperature, and the participants with high doses have exhibited greater immune response against the virus (Edible Cholera Vaccine Made of Powdered Rice | EurekAlert! n.d.; see also chapter "Delivery of Drugs and Vaccines Through Plant Molecular Farming").

1.2.3.2 Industrial Proteins

Industrial proteins are proteins that are often used for industrial application in huge quantities and are required to be cost effective for production. The proteins include enzymes and diagnostic proteins (Hood and Woodard 2002; Xu et al. 2018). The plant expression system is preferred due to the cost-efficient process in the production of the industrial enzyme, the high protein expression level of protein of interest at a specific region of the plant, and the easy harvesting process (Hood and Woodard 2002). Among various available transgenic plants, field plants are the most preferred type to produce industrial proteins. This is because of the numerous benefits it possesses, including being cost effective, ability to store the protein at the desired organ to achieve high stability, and fast production (Xu et al. 2018). One of the first-ever proteins produced for industrial purposes was avidin developed and sold in 1998. One factor that bothered the production of avidin was that the stability of protein produced after ten generations became lower and insufficient. Over time, the researchers overcame this by the selection process and modification in germplasm to maintain the stability of avidin for multiple generations without any depletion (Hood and Woodard 2002; Xu et al. 2018). Some of the studied and available commercialized proteins and enzymes include hydrolases, cellulase, α-amylase, β-glucuronidase, and trypsin (Xu et al. 2018). One of the famous and widely used enzymes in industries is papain derived from the papaya fruit. It is widely applied in the food industry, where it is a frequently used type of protease to tenderize the meat (Shouket et al. 2020; see also chapter "Molecular Farming of Industrial Enzymes: Products and Applications").

1.2.3.3 Therapeutic Proteins

Proteins are one of the important biological molecules which can be categorized based on their function and pharmacological activity. Therapeutic proteins are proteins that can be found naturally in the human body and can be uniquely designed for use in pharmaceutics and treatment purposes. Therapeutic proteins can be classified according to their molecular types, and some of the examples include monoclonal antibody, blood factors, growth factors, enzymes, hormones, interferons, and interleukins (Awwad et al. 2018; Dimitrov 2012). The requirement and need for these therapeutic proteins are increasing annually, and the conventional production of these proteins is time consuming and very expensive. The plant's expression

system is used to overcome these issues, where plants can produce these proteins in large amounts, at the same time being low-cost, safer, and stable protein compared to those produced in mammalian cells or other expression systems (Xu et al. 2018). Monoclonal antibody (mAb) is one of the most known therapeutic proteins, where it is produced from B cells-lymphocytes and is known to replace antibodies in the host. mAb can improve, modify, and mimic the host immune system to attack pathogens or cancerous cells (Monoclonal Antibody Drugs for Cancer: How They Work—Mayo Clinic n.d.; Tabll et al. 2021). One of the studies performed by Lai et al. has proven that mAb developed from the plant system cures West Nile virus infection. The transgenic tobacco plant has been designed to produce humanized mAb that has a high affinity towards West Nile virus envelope protein, which results in viral fusion inhibition (Lai et al. 2010). The purity of the mAb produced via the transgenic tobacco plant is more than 95%, and the neutralization activity of the mAb was noticeably high (Lai et al. 2010). Another type of therapeutic protein produced using a plant system is the growth factor, where it is a released bioactive compound that can influence cell development and growth (Stone et al. 2021). One of the studies conducted by Musiychuk et al. has produced human erythropoietin growth factors from the plant system to promote hematopoietic stem cell differentiation to red blood cells. They also analyzed another three human growth factors, which are stem cell factor, interleukin, and insulin-like growth factor-1, using transgenic tobacco plant. The researchers have used the tobacco mosaic virus vector approach to introduce the desired gene to produce these growth factors. The EC50 values of these four human growth factors were substantially high and showed promising results (Musiychuk et al. 2013).

1.3 Transformation Strategies

1.3.1 Nuclear Transformation

Stable nuclear transformation is the most common and widely used method to date for genetic manipulation in plants to produce recombinant protein (Horn et al. 2004; Shanmugaraj et al. 2020). Nuclear transformation is the introduction of the genes of interest into the nuclear genome of plants to cause the alteration of genetic structures and ultimately express the transgene within the host genome (Alireza and Nader 2015). The foreign gene can be incorporated into the in vitro plantlets in a stable manner as a transgene in the plant expression vector by *Agrobacterium tumefaciens*-mediated transformation or particle bombardment, biolistic method (Horn et al. 2004). *Nicotiana* genus is widely used as the expression system to produce most of the recombinant proteins due to its rapid growth rate and easy manipulation of the gene. Furthermore, crops such as rice, maize, tomato, and potato also showed their potential as expression systems in PMF (Burnett and Burnett 2020). Upon the development of the pool of transgenic lines, the best transgenic line will

be screened for protein production. The succession of the integration of transgene into the plant genome would result in the recombinant protein production in successive generations (Shanmugaraj et al. 2020).

This method has been used to produce the largest number of recombinant proteins in PMF. The recombinant proteins such as erythropoietin, collagen, and human-secreted alkaline phosphatase were produced via *Agrobacterium*-mediated method with tobacco as the expression system (Komarnytsky et al. 2000; Matsumoto et al. 1995; Ruggiero et al. 2000). Human growth hormone (hGH) and α1-antitrypsin were produced via biolistic method with rice as the expression system (Kim et al. 2008; Terashima et al. 1999). Exploitation of this method has shown the advantage to perform in crop species such as grains, rice, cereals, and corn. As the protein products accumulate in the seed, it can be harvested in a dry condition and stored for a long term until the accomplishment of processing (Delaney 2002). However, the potential of some grains to cross with the native species or food products has affected the social acceptance towards the use of this method (Commandeur et al. 2003).

1.3.2 Plastid Transformation

Plant cells contain three DNA-harboring organelles, which are the nucleus, plastid, and mitochondria (Yu et al. 2020). The success in engineering the genome of chloroplast for disease, herbicides, and insect resistance as well as for the production of biopharmaceuticals had led to a new era of plant biotechnology to a more environmental-friendly direction (Daniell et al. 1998). This is because the genome of chloroplast defied the laws of Mendelian inheritance that resulted in avoiding the outcrossing of transgenes with native species and reducing the toxicity of transgenic pollen to the non-targeted insects (Cosa et al. 2001; Svab et al. 1990). The genome of plastids is a circular double-stranded DNA that is inherited maternally and presents in many copies in the organelle (Adem et al. 2017). Thus, it is protected from gene silencing, which would result in lowering the transgene expression and thereby allowing the accumulation of high level of foreign protein, 5–40% of total soluble protein (Roudsari et al. 2009).

Svab et al. were the first to describe the system for plastid transformation using higher plant, tobacco, after the success of plastid transformation on unicellular algae, *Chlamydomonas reinhardtii*. Chloroplast genetic engineering started to develop in 1980s where isolated intact chloroplasts were able to introduce into protoplasts (Svab et al. 1990). The crop species that can be used for plastid transformations include tobacco, tomato, potato, eggplant, and soybean (Ding et al. 2006; Ma et al. 2015; Shanmugaraj et al. 2020; Zhou et al. 2004). There are three main steps in plastid transformation as illustrated in Fig. 1.2. Firstly, the transgene is delivered to cells of an explant. Secondly, the transgene is integrated into the chloroplast genome by going through homologous recombination at a specific site (Yu et al. 2020). Lastly, the ideal transformants are screened repeatedly and thoroughly on

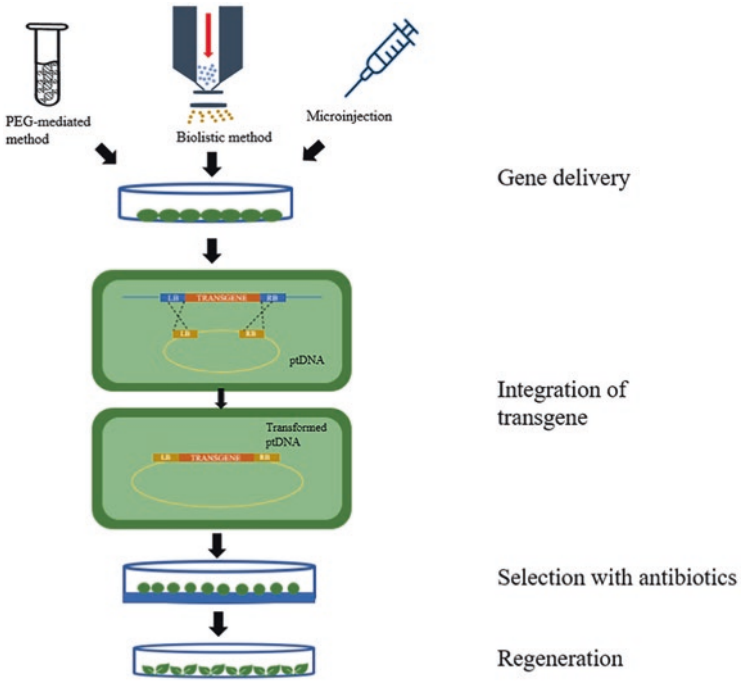

Fig. 1.2 Plastid transformation process

selection medium until a state of homoplasmy (fully eliminate the wild-type genome in the pool) (Lu et al. 2006). The selection process can be conducted on the medium containing spectinomycin (Alireza and Nader 2015). The positive transformants will then be regenerated into a stable transgenic line.

Biolistic transformation and polyethylene glycol (PEG)-mediated transfection are the two most common methods for introducing the foreign DNA into chloroplasts (Lu et al. 2006; Yu et al. 2020). PEG-mediated method only works on protoplasts by culturing the protoplasts with the presence of PEG vesicles containing foreign DNA to allow the DNA uptake by protoplasts (Lu et al. 2006). However, the development of gene gun by John Sanford to be used as a transformation device allows direct transformation to the plant cells instead of isolating protoplasts (Daniell 1993). Recently, nanoparticles were introduced as a new strategy for plastid transformation to allow the introduction of DNA without the need of other instrument or isolation of protoplasts (Kwak et al. 2019).

Antigen vaccines, protein-based drugs, and industrial enzymes are able to be produced from the chloroplast of the plant via plastid transformation (Yu et al. 2020). Protein-based drug insulin is able to be produced in transplastomic tobacco plants with the expression of 14.3% of total soluble protein (Kwon et al. 2013). This might help to solve the issues of high cost of insulin produced by yeast due to the additional cost needed to maintain the yeast suspension (Daniell et al. 2016).

Besides, plant chloroplast is able to perform a proper posttranslational modification of insulin to promote the proper folding like phosphorylation and disulfide bond formation (Řepková 2010).

The culprit of cervical cancer is human papillomavirus (HPV), which kills more than 250,000 of women annually. The protein E7 antigen from HPV type 16 (HPV-16 E7) has been selected as a candidate to produce therapeutic vaccine (Morgenfeld et al. 2009, 2014). It has been reported that HPV-16 E7 can be expressed in the tobacco plant via plastid transformation (Morgenfeld et al. 2009). Studies also showed that mice and rabbits developed specific immune responses after being injected with chloroplast-derived vaccines (Molina et al. 2005). However, the accumulation of E7 protein in the chloroplast is relatively low due to the instability of natively unfolded protein. Thus, the strategy of fusing E7 coding sequence with potato virus X coat protein was done to improve the stability and immunogenicity of the antigen (Morgenfeld et al. 2014; see also chapter " Development of Oral Prophylactic and Therapeutic Vaccines Against HPV on the Basis of Plant Expression System").

In addition, genetically engineered chloroplast genome can be used to produce biomaterials. The biodegradable polyester biopolymers such as polyhydroxyalkanoates (PHAs) are a perfect alternative to petroleum-based plastics that are naturally synthesized by microorganisms (Svab et al. 1990). However, transgenic crop via plastid transformation like tobacco has the potential to be used as a source of PHAs. Transgenic crops can achieve an accumulation level of PHA up to 40% dry cell weight of the leaf and 20% dry cell weight of the seed (Dobrogojski et al. 2018). Industrial enzymes such as β-glucosidase and xylanase can be obtained via chloroplast production in tobacco plants as well (Jin et al. 2011; Kolotilin et al. 2013).

Despite the great potential of plastid transformation, only tobacco is practically possible for this method. However, due to the toxicity of the plant with full of poisonous alkaloid, the use of tobacco in the production of edible recombinant protein or vaccine is not feasible (Horn et al. 2004; see also Chap. 9).

1.3.3 Agroinfiltration

Agroinfiltration is based on the *Agrobacterium*-based approach for transient transformation that is commonly used to transfer the foreign gene into somatic cells of targeted plants such as leaves (Debler et al. 2021; Kaur et al. 2021). Early discovery of *Agrobacterium tumefaciens* in the late eighteenth century has played a prominent role in plant biotechnology. Initially, it was developed to use as a tool for the investigation of plant-virus interaction (Chen et al. 2013; Kaur et al. 2021). However, the first transgenic plant produced via *Agrobacterium*-mediated transformation process in the year 1983 opened a door for plant genetic transformation for plant farming interventions (Ramkumar et al. 2020). In addition, *Agrobacterium*-mediated transformation is a relatively effective, simple, and less time-consuming transient expression method compared to biolistic method and PEG-mediated transfection of

protoplast as it does not involve the isolation of protoplast (Belhaj et al. 2013; Norkunas et al. 2018). Agroinfiltration works by the principle of delivering the gene of interest carried by recombinant *Agrobacterium* in the extracellular spaces of the intact leaves via physical or vacuum infiltration (Donini and Marusic 2019). It is preferable because this method has high level of expression, which yields up to 50% of TSP compared to stable transformation, which only yields less than 1% of total TSP. Human growth hormone (hGH), aprotinin, and human fibroblast growth factor 8b are the examples of transient expression application (Sheludko 2008).

It is an indirect gene delivery method, in which *Agrobacteria* species has the ability to transfer DNA into the plant cells. *Agrobacterium tumefaciens* is a pathogen of the plant. It has a tumor-inducing (Ti) plasmid containing transfer DNA (T-DNA) that is able to transfer into plant host cells by infecting the plant cells (Chen et al. 2013). It would result in causing tumors and crown gall in the plant as Ti plasmid is the factor of tumorigenesis and part of the plasmids, and T-DNA was incorporated into the plant genome (Gelvin 2003). On the other hand, *Agrobacterium rhizogenes* harbors root-inducing (Ri) plasmid that induces abnormal root growth (Hwang et al. 2017). Therefore, it was then proposed that Ti or Ri plasmids can be used as a vector to introduce transgene into plant cells by developing the *Agrobacterium* strains that do not cause tumor formation but have the capability to transfer foreign gene (Chen et al. 2013; Gelvin 2003). T-DNA and virulence region are the two genetic components that are responsible for the transformation (Chen et al. 2013).

There are few plant hosts that could be used including *Lactuca sativa* (Lai et al. 2012), *Nicotiana tabacum*, *Arabidopsis thaliana*, *Glycine max* (King et al. 2015), and *Pisum sativum* (Guy et al. 2016). However, *Nicotiana benthamiana* is the most preferred experimental host due to the high susceptibility to a diverse range of viruses and other plant-pathogenic agents including bacteria, oomycetes, and fungi (Goodin et al. 2008). In addition, *Nicotiana benthamiana* has gained a great attention to serve as "biofactories" in producing recombinant proteins due to its ability to perform posttranslational modifications for appropriate protein folding to result in functional biological activity (Bally et al. 2018). The expression of the transgene could be controlled by a suitable promoter such as cauliflower mosaic virus 35S (CAMV-35S) (Debler et al. 2021). However, it is not easy to clone a foreign gene into T-DNA due to large size of Ti plasmids and lack of unique restriction endonuclease cleavage sites on T-DNA and elsewhere (Gelvin 2003). Thus, two approaches were developed to facilitate the introduction of foreign gene into T-DNA including co-integrating system and binary vector system. But binary vector system is the most performed method of molecular genetic modification (Mardanova et al. 2017).

Co-integrating system generally refers to cloning the gene by an indirect mean into Ti plasmid in a way that the new gene was in *cis* with the virulence genes resulted on the same plasmid (Gelvin 2003). A region of DNA containing unique restriction endonuclease sites targeted for disruption is cloned into a broad host range plasmid, which is able to replicate in *E. coli* and in *Agrobacterium*. It is known as an intermediate plasmid. The foreign gene with antibiotic resistance marker is cloned into a unique restriction endonuclease site. A disarmed Ti plasmid contains

the vir function maintained in *Agrobacterium*. The intermediate plasmid is then conjugated with *Agrobacterium* containing disarmed Ti plasmid and undergoes homologous recombination. *Agrobacterium* with co-integrated plasmid is selected and introduced into the plant (Garfinkel et al. 1981; Gelvin 2003). However, the protocols are sophisticated and therefore binary system is generally a preferred method in agroinfiltration.

Binary vector system revolutionized the *Agrobacterium*-mediated transformation method (Gelvin 2003). pGreenII is one of the most widely used binary vectors (Hellens et al. 2000). It generally refers to the system in which *vir* and T-DNA regions of the Ti plasmids can be split into two separate replicons but located in the same *Agrobacterium* cell (Simpson et al. 1986). Therefore, this system consisted of two plasmids: T-binary vector and the vir helper plasmid. T-DNA region containing foreign gene is located on the binary vector, and non-T-DNA region contains the origins of replication that could perform replication in both *E. coli* and in *Agrobacterium* as well as antibiotic resistance genes for selection process (Komori et al. 2007). It is also known as a small artificial T-DNA since this vector does not contain tumor-inducing gene and vir genes, and thus it has smaller size compared to Ti plasmids. The vir gene containing replicon is served as a helper plasmid to synthesize vir protein (Hwang et al. 2017). The vir region serves a function in participating in the events in the host cell that involve cytoplasmic trafficking of T-DNA, nuclear targeting, and integration into host genome as well as improvement of the virulence (Howard et al. 1992). As a result, the foreign gene could be easily introduced to small T-DNA regions within binary vectors. Then, the characterization and verification of the constructs are done in *E. coli*. T-binary vector is able to mobilize into *Agrobacterium* strain containing vir helper plasmids. This system has simplified the protocol in generating transgenic plants compared to co-integrating system (Lee and Gelvin 2008). The schematic diagram of co-integrating vector and binary vector is illustrated in Fig. 1.3.

Syringe infiltration and vacuum infiltration are the two most common agroinfiltration methods (Kaur et al. 2021). Syringe infiltration involves the use of needleless syringe to introduce *Agrobacterium* into the leaves of the plant, and Tween-20 could be used to improve the efficacy (Zhao et al. 2017). *Agrobacterium* together with infiltration medium is injected into the nick-created leaves. It can either transfer a target gene into the entire leaf entities or introduce multiple genes in different areas of a leaf for multiple assay purposes (Vaghchhipawala et al. 2011). On the other hand, vacuum infiltration is done by submerging the plant leaves into the infiltration media containing *Agrobacterium* in a vacuum chamber under a negative atmospheric pressure (Simmons et al. 2009). The air of interstitial spaces of the leaves is drawn out by vacuum, and air filled by *Agrobacterium*-containing media occupies the spaces when the vacuum is released to achieve agroinfiltration (Chen et al. 2013). Vacuum filtration is more robust compared to syringe filtration as large number of plants can be infiltrated in a shorter time and it can be used for those plants that cannot be infiltrated by syringe (Rivera et al. 2012). According to the studies, vacuum infiltration requires a shorter timeframe for infiltration, which only takes 3 min for the process, compared to syringe infiltration, which needs 15 min

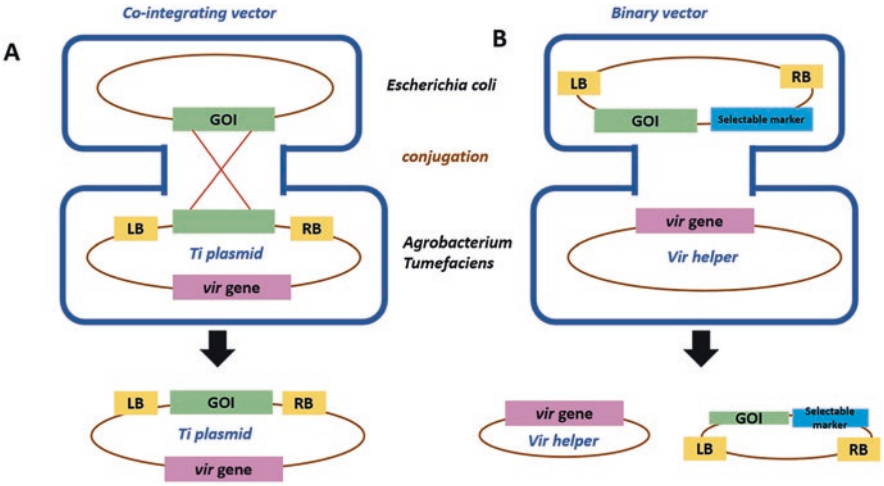

Fig. 1.3 Agroinfiltration process. *GOI* gene of interest, *LB* left border, *RB* right border

(Leuzinger et al. 2013). Hence, this method has facilitated the development of a protein production platform that is fast, safe, and economical.

To date, *Agrobacterium*-mediated transient expression has considerably drawn the attention of scientists as an approach for large-scale production of recombinant protein using plant systems due to its effectiveness and safety (Sheludko 2008). It has been practically utilized for the transient expression of recombinant protein. Furthermore, agroinfiltration is also applicable for a virulence gene discovery and various studies in plant: gene silencing, resistance mechanism, promoter characterization, vaccine production, as well as phyto-sensing studies (Chen et al. 2013; Sheludko 2008).

1.3.4 Viral Infection

Transient expression based on virus-based vectors is another alternative to complement transgenic plants, which offers high expression level of protein in the time of few days (Shanmugaraj et al. 2020). Plant viruses are not just used as pathogens but had evolved to be used as a tool for recombinant protein expression that is of industrial importance such as vaccine antigens and antibodies (Yuri Gleba et al. 2007; Ibrahim et al. 2019). It has also been used for chimeric viral vaccine production and as an agent of nanoparticles for drug delivery (Ibrahim et al. 2019). Tobacco mosaic viruses (TMVs) and X potato viruses are the most prominent viruses to be used as a basic tool for protein production in PMF (Alireza and Nader 2015; Bamogo et al. 2019). The general approach is that the viruses are engineered to contain a gene of interest upon introduction into the host and that will replicate in the host and produce the recombinant protein in a significant amount in infected plants (Bamogo

et al. 2019; Gleba et al. 2007). The plant viruses can be engineered to result in expressing subunit vaccines or to serve as epitope presentation systems (Bamogo et al. 2019). The viral infection processes are illustrated in Fig. 1.4.

Ever since the emergence of virology, TMV was the first exploited virus to be developed as a viral vector to produce various and diverse types of recombinant proteins and became the most prominent choice to develop natural vectors (Ibrahim et al. 2019; Kagale et al. 2012). It began with a Russian scientist, Dmitri Ivanovsky, who described that infected sap of leaves with tobacco mosaic diseases retained the infectious properties (Lustig and Levine 1992) with the ability to replicate within the plant cells even after extracted sap had passed through bacteria-retaining filters since the filtrate is infectious to infect tobacco plants (Van Regenmortel 2008). It was then followed by the success of precipitating the pathogen of tobacco mosaic disease in 1927, which came to reveal that the pathogen contained RNA as well as protein DNA, which is the TMV (Rifkind and Freeman 2005). TMV belongs to *Tobamovirus* genus with a single-stranded RNA and a positive sense (Dunigan and Zaitlin 1990). Viruses with RNA genome are preferably to be exploited due to the ability of the virus to build the capsid around the genome, which results in less constraint on the size of transgene insertion (Yusibov et al. 2006). Within the untranslated region of the genome, it harbored transfer RNA like structure that encoded for protein needed for RNA replication, movement protein as well as coat protein (Ibrahim et al. 2019). The promoter of viral RNA can be manipulated to synthesize the recombinant messenger RNAs in the whole plant. Immunogenic molecules could be expressed using TMV vectors such as anti-idiotypic single-chain variable fragment (scFv) antibodies, and the antibodies were shown to be protective against

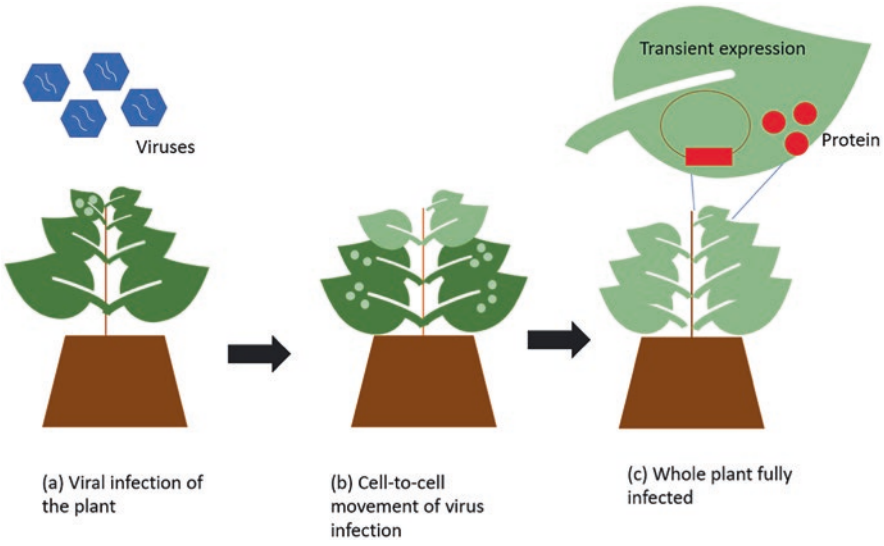

Fig. 1.4 Viral infection process

cancer by recognizing the immunoglobulin on human tumor cells (McCormick et al. 2003).

Potato virus X (PVX) vectors were developed for transient expression of foreign genes in plants in 1992 (Chapman et al. 1992). It is a positive-sense RNA plant virus with flexuous and rod shape. The molecular event of PVX infection had paved the way in developing expression vectors, and it is ultimately applied in functional genomics studies as well as biosynthesis of heterologous recombinant protein (Lico et al. 2015). The strategy is that the foreign gene is inserted between a duplication of the subgenomic promoter sequence directed at the production of mRNA, in which the coat protein is translated (Lacomme and Chapman 2008). The capsid protein gene and triple-gene block are engineered to deconstruct vectors. PVX can be used to express full-length proteins, fusion proteins, as well as epitope for the use in medical purpose (Bamogo et al. 2019).

These plant viral vectors can be categorized into different categories based on the manner in which they are designed. The first-generation vector, also the first strategy, is the "full virus strategy" which uses the unmodified native virus to maintain its properties completely. Hence, this strategy is to design the viruses that are fully functional and retain infectivity and virulence despite carrying and expressing heterologous protein sequences (Awram et al. 2002; Gleba et al. 2004). However, due to the limitation of the first-generation vector and safety issues that can spread in the environment, another strategy called "deconstructed virus strategy" has been established in which the deconstructed vectors are composed solely of the genome that is required for virus replication by removing unnecessary and retaining only the essential and required part of the vector to function (Gleba et al. 2014; Pogue et al. 2002). This would also allow large size of the gene to be incorporated into the genome of virus and with high level of protein expression since the protein coded for unnecessary open reading frame was deleted and replaced by the gene of interest (Bamogo et al. 2019; see also chapter "Plant Viral Vectors: Important Tools for Biologics Production").

1.3.5 Magnifection System

Magnifection system, magnICON technology, was developed by a German plant biotechnology company named Icon Genetics that utilized the advantages of a "deconstructed virus" strategy that lacks the ability to infect other plants and combine with agroinfiltration for the gene delivery (Gleba et al. 2005; Hefferon 2017; Marillonnet et al. 2005). Magnifection system is a scalable protocol for recombinant protein production in plant that relies on the transient amplification of the deconstructed viral vectors, which are delivered by *Agrobacterium* to different areas of a plant body (Gleba et al. 2005). It is a new generation of transfection that effectively addresses most of the drawbacks of other available transformation technologies in PMF. It could be used to produce recombinant antigen and antibodies in *Nicotiana benthamiana* (Klimyuk et al. 2012). This technology has combined the

advantages of three biological systems: vector efficiency, ability of *Agrobacterium* in its systemic delivery, and rate and expression level of a virus as well as the post-translational capabilities (Gleba et al. 2005; Hiatt and Pauly 2006).

The general approach of this technology is to supply a plant cell with an RNA or linear DNA molecule by *Agrobacterium* that assembles inside the cells assisted by the site-specific recombinase instead of supplying a complete viral vector mature viral particle (Giritch et al. 2006). Thus, in vitro transcription in generating RNA-based vectors can be avoided as engineered in vitro RNA synthesis is the time and effort bottleneck of functional genomics using viral vectors for the actual expression (Fitzmaurice et al. 2002). *Agrobacterium* carrying T-DNAs encoding for RNA replicons is prepared in a diluted suspension in order to infiltrate into the plant. The bacteria generally function as a primary infection to the plant for systemic movement. On the other hand, the viral vectors are responsible for cell-to-cell spread, amplification, as well as high-level expression of recombinant protein (Marillonnet et al. 2004). However, the coat proteins of the virus strains are removed to prevent its systemic delivery to infect the whole plant (Alireza and Nader 2015). This approach is preferable because it provides very high yield of protein with up to 80% of total soluble protein with a very short period of expression, which takes up to 3–4 days, and most importantly, it is inherently scalable (Marillonnet et al. 2004).

This new technology can be applied for the plant vaccine production. A deconstructed TMV vector is employed to generate E7 oncoprotein of human papillomavirus (HPV) (Plchova et al. 2011) as well as M2e epitope of influenza (Denis et al. 2008). Then, the deconstructed TMV vector that generates antigen is housed within an *Agrobacterium tumefaciens* binary vector that would result in generating vaccines against influenza virus and cholera. Furthermore, magnICON deconstructed vector is one of the vectors that are used to develop personalized medicine against non-Hodgkin's lymphoma (NHL) (McCormick et al. 2008). NHL occurs due to the overproliferation of B cells that present a unique cell surface idiotype that is only specific to that individual (Singh et al. 2020). Hence, NHL can be vaccinated with their own idiotype. Thus, personalized medicine can be generated using the magnifection method. Deconstructed magnICON vectors express the TMV constructs composed of scFv subunit and full-length idiotype Ig molecules as heavy and light chains that will be assembled into full immunoglobulins in the plant. The vaccine constructs have successfully passed phase I clinical trial in which they have been demonstrated to be safe and elicit few adverse effects (Hefferon 2017; see also chapter "Antigen-Specific Immunotherapy for Allergic and Autoimmune Diseases Using Plant-Made Antigens").

As the summary, Table 1.2 highlights the advantages and disadvantages of the five main transformation strategies used in plant molecular farming.

Table 1.2 Advantages and disadvantages of transformation strategies

Transformation strategy	Advantages	Disadvantages	References
Nuclear transformation	• Simple and widely used method in many plant species • Reduced cost of storage • Increased capacity of scale-up • Has unique glycosylation pattern	• Low levels of expression • Risk of gene silencing • Possibility of transgene contamination	Burnett and Burnett (2020), Moon et al. (2020)
Plastid transformation	• Transgene containment • High levels of expression • High stability and reduced risk of gene silencing • Ease in ecological controls	• Lack of glycosylation in plastids • Lack of field release of transgenic plant	Gleba and Giritch (2012), Maliga (2017)
Agroinfiltration	• Shorter timeline of process • Increased expression of recombinant protein • Potential of introducing more than one T-DNA into plant cells	• Challenges in scalability • Some plant species are not compatible with *Agrobacterium* • Some plant species are not amenable to agroinfiltration • Challenges in RNA silencing due to the host recognizing T-DNA as foreign	Canto (2016), Menassa et al. (2012)
Viral infection	• Increased expression of recombinant protein • Allows the screening of multiple construct variants in various plant species • Capability of expressing targeted genes at a specific stage of plant growth by differing inoculation timing • Small size of viral genome and ease in manipulation • Shorter timeline of process than stable transformation strategies	• Gene of interest introduced could be lost due to mutation or deletion over time • Chances of adverse effects on plant host or interaction of plant viral vector with other viruses • Chances of transmission to the environment	Abrahamian et al. (2020), Cañizares et al. (2005)

(continued)

Table 1.2 (continued)

Transformation strategy	Advantages	Disadvantages	References
Magnifection system	• Shorter timeline of process • Increased expression of recombinant protein • High yield of protein reduces downstream processing cost • Versatility in genes expressed • Permits the co-expression of several proteins that are required for hetero-oligomeric protein assembly • Vector efficiency	• Challenges in expressing an economically acceptable level of gene as high level of expression of certain proteins may be toxic to host plant • Challenges in expressing oligomultimeric proteins as the manipulation of viral vector requires polypeptides to be expressed in equimolar	Burnett and Burnett (2020), Gleba et al. (2005)

1.4 Advantages of PMF

Given the diversity in protein expression systems that are currently available, there is no surprise that each system's characteristics are compared head-to-head to determine their functions and suitability of use. While other expression systems, such as mammalian cell lines, bacteria, yeast, and insects, certainly have their niche advantages, plants often take the upper hand when it comes to molecular farming.

Often, the first factor to be brought up for discussion is the commerciality of plants in molecular farming. The cost of operations is essential for sustainable production of recombinant proteins. Molecular farming on an industrial scale using cell lines, inclusive of mammalian, bacterial, or fungal (yeast), relies on using large fermenters, bioreactors, and other equipment, which contributes to its high cost (Alireza and Nader 2015; Fischer and Schillberg 2016). In contrast, the cost of PMF is relatively cheaper compared to other expression systems as transgenic plants can be grown in greenhouses or vertical farms, allowing PMF to reach agricultural scalability and space efficiency due to the ease of growing additional plants (Buyel 2019; Fischer and Schillberg 2016). Hence, this lets PMF to have a relatively higher production ceiling and ease of manufacturing. Running costs such as the expensive media typically used in cell cultures, especially in mammalian cell expression systems, are also much higher compared to the defined fertilizer solutions for cultivating plants (Buyel and Fischer 2012; Xu et al. 2017).

The ease of storage and transportation of PMF products drives its costs lower. The robustness and durability of plants result in lower storage requirements, which also consequently reduces costs for transportation. Additionally, the utilization of plants in molecular farming allows the manufacture process of recombinant proteins to be conducted on-site (through the use of greenhouses and farms, as mentioned previously), skipping the requirements of existing or constructing new

laboratory facilities (Chung et al. 2021). This not only reduces transport requirements, but would also provide accessibility to rural regions with pressing needs for biopharmaceuticals. Utilizing PMF for rapid deployment of emergency medicines or vaccines was shown to be possible during the 2014 Ebola outbreak in West Africa where ZMapp, an experimental treatment for Ebola produced via transient expression in *N. benthamiana*, received authorization for emergency deployment before clinical trials were conducted (Schillberg and Finnern 2021; Zhang et al. 2014). As such, many look to PMF as a potential pandemic response that may be able to help combat the ongoing COVID-19 pandemic (Kumar et al. 2021; Leblanc et al. 2021; Tusé et al. 2020).

The safety of recombinant proteins and molecular farmed products is a highly controversial issue that concerns consumers, researchers, and investors alike. Plants, which are innately immune to most human pathogens and endotoxins, present little to no risk of contamination during the cultivation of transgenic plants for PMF (Alireza and Nader 2015; Shanmugaraj et al. 2020; Wang and Ma 2012). This lends credibility of PMF products' relative safety compared to other expression systems such as bacteria or yeast. The manufacturing process is also simplified using plants when PMF produces edible products in the forms of fruits and vegetables. In these cases, post-processing and purification steps can be skipped almost entirely during manufacturing (Wang and Ma 2012). Not only would this be beneficial by reducing production costs, but it also allows convenient oral administration to consumers.

Additionally, plant recombinant proteins are able to undergo relatively complex posttranslational modifications, such as glycosylation, phosphorylation, and disulfide bridging, enabling further complex proteins to be produced in PMF. This gives plants a further edge compared to bacterial, fungal, or insect expression systems, where only simple modifications can be performed, or none at all. That said, plants still fall short compared to mammalian cell lines in terms of the similarity of modifications to humans. The differences in posttranslational modifications, such as differing glycosylation patterns or chemically different sugars involved, may lead to immunogenicity or loss of function of the protein (Marintcheva 2018). Solutions to overcome this problem involve expressing relevant mammalian posttranslational modification enzymes in the plant system itself and inversely silencing the relevant plant systems that are still in development (Marintcheva 2018).

1.5 Challenges of PMF

Although PMF approach is advantageous in many aspects, there exists a bottleneck for the utilization of PMF in the production of recombinant proteins commercially (Schillberg and Finnern 2021; Xu et al. 2018). One of the major limitations of PMF is its low productivity, which is perceived to limit its successful commercialization. The levels of recombinant proteins produced are reported to hardly exceed 100 μg/kg of the plant tissue fresh weight or 100 μg/L of plant cell suspension cultures (Permyakova et al. 2021; Schillberg and Finnern 2021). This level of recombinant

proteins produced in plant expression systems are relatively considered to be much lower than other expression systems. However, plants are able to yield a higher biomass when grown in the field compared to conventional expression platforms such as mammalian cells, bacteria, and yeast that involve fermentation (Twyman et al. 2013). The yield produced by the plants can be affected by a number of factors including environmental factors such as temperature, humidity, and light; protein targeting strategies; expression strategies; and protease inhibitor co-expressions (Schillberg et al. 2019).

Although challenges in the expression of transcript have been identified, there are ways of optimizing the level of transcriptions. A commonly utilized approach is the utilization of cauliflower mosaic virus (CaMV) 35S promoter, which is suitable for dicotyledonous plants, and maize-1 ubiquitin promoter, which is suitable for monocotyledonous plants (Alireza and Nader 2015). Apart from the promoter, the polyadenylation site also plays an essential role in increasing the transcription levels, and some of the commonly used polyadenylation sites are from CaMV 35S transcript, pea SSU gene, and *Agrobacterium tumefaciens* nopaline synthase gene (Singh et al. 2021). The silencing or degradation of RNA may also affect the stability of gene transcripts, which leads to a lower recombinant protein yield (Alireza and Nader 2015). In terms of maintaining stable activity of recombinant proteins in transgenic plants, targeting transgenes to be expressed in its storage organs such as tubers or seeds is beneficial (Moustafa et al. 2016). In fact, activity of human coagulation factor IX protein, which was expressed in the seeds of soybean plants, was reported to be functionally stable for a period of 6 years (Moustafa et al. 2016; see also Chaps. 5 and 6).

Glycosylation refers to covalent binding of various glycans such as saccharides, sugar, or carbohydrates to a protein (Li et al. 2019). This posttranslational modification is essential for the function, stability, and activity of proteins. Although plants are advantageous compared to bacteria and yeast in this aspect, the difference in the carbohydrate pattern of glycosylation in plants when compared to mammalian cells is a challenge. The glycosylation pattern that occurs in plants may give rise to glycan-specific antibodies and subsequent undesirable clearance of the plant-made therapeutics when administered (Gomord et al. 2010; Schoberer and Strasser 2018). The N-glycan glycoprotein epitopes including Le^a, α-1,3-fucose-containing, and β-1,3-xylose-containing epitopes are typically immunogenic (Gomord et al. 2010). Apart from these, the selection of a suitable plant species as the host is also critical. Although there are various options for a host, each plant has its advantages and disadvantages (which was discussed in previous sections). The phenol and toxic alkaloid contents, amount of biomass, and tight regulatory requirements for certain plant species should be taken into consideration when selecting a host (Burnett and Burnett 2020).

Another major hurdle in PMF is challenges with regulatory approval and social acceptance. PMF products are managed by different regulations in different parts of the world based on its risk assessments. Concerns regarding PMF that have been raised include the risk of contamination of feed or food crops that may pose a threat to food industries and farmers, the risk of possible horizontal gene transfer which

may result in the development of antibiotic-resistant microbes, and the risk of transgene spread or unwanted exposures to wild-type plants (Obembe et al. 2011). Hence, biosafety strategies and regulations are essential. The need to follow good manufacturing practice (GMP) guidelines that have been set based on mammalian and bacterial cell expression systems instead of developing specific guidelines for plants is known to be challenging (Menary et al. 2020). In the USA, the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service and the Food and Drug Administration (FDA) are key regulatory bodies that regulate the cultivation of plants and the safety of pharmacological products, respectively (Breyer et al. 2009; see also Chaps. 14 and 15).

1.6 Concluding Remarks

PMF has shown to possess great potential to be utilized for a variety of applications. Plants including tobacco, cereals, fruits, and vegetables have been utilized as bioreactors successfully. With an increase in the number of studies investigating the potential of PMF, various stable and transient transformation strategies have been developed. Utilizing plants as expression hosts is suggested to greatly reduce the production and transportation cost of recombinant proteins. With these advantages comes challenges in terms of productivity. However, the development of optimization strategies has enabled successful PMF utilization, and it would promise various potential applications in the future.

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