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Vaccination and Biopharming Technology

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Abstract

Vaccination is very essential medical aid to prevent the onset of disease with the development of science and technology many advances occur in vaccination technology biopharming technology is most recent technology serving as a platform for vaccine development plant vaccine either in the form of capsules or injections presently and edibles for the future are most preferable for under developed countries and automated biopharming factories are working in this respect, the expression of proteins depends on the selected host system, intrinsic properties of desired protein. Nuclear transformation is most suitable and well-studied of all, biolistic transformation technology removes the need of vectors, but targeting is compromised. Stability of recombinant DNA can be maintained by over-expression of desired protein and optimization is achieved by the selection of suitable promoter, expression either stable or transient have utility. Finishing is done by downstream processing maximum protein extraction, cost-effective processing with purification is yet a goal to be achieved.

Keywords: Vaccines; Plant factories; Biopharming; Recombinant proteins; Host system; Expression; Transformation

Introduction

Vaccination is the only way to get immunity before the onset of disease, vaccine consists of biological preparation that generates the immunity to a specific disease it may contain killed or weakened version of microbes or sometimes the disease is caused by the toxins produced by microbes, the vaccine contain toxin or a part of pathogen but not the whole microbe is called toxoid [1]. Subunit vaccines are getting popularity, specific proteins or the genes coding for disease-causing proteins are isolated from the disease-causing agent and introduced into another virus, conjugate vaccines are like subunit vaccines contain fragments from the coat of bacteria, which are chemically linked to a carrier protein. With the discovery of virus-like particles, the process of vaccine development further simplifies, single virus protein is isolated from different strains the expression of these proteins results in the creation of VLP [2]. They do not contain genetic material from viruses and so, cannot cause disease but incite the immune response. VLP for HBV, NV, and HPV have been produced in plants [3,4]. VLP is gaining interest because of their favorably compact structure which may allow them to withstand severe conditions gastrointestinal environment [5,6]. In the present review, our focus is on plant vaccines and their production through molecular pharming technology. Pakistan is a developing country, a report by UNDP Pakistan and OPHI, University of Oxford, 2016 shows that 4 out 10 Pakistani's life in multidimensional poverty, which makes 39% of total population common public cannot afford high cost of medical treatments and vaccination, according to another report by World health organization and UNICEF 2016 reveals that 70-80% of children are vaccinated and DTPcv3 coverage decreases from 2010 to 2016 from 82% to 72% in Pakistan by 2016. Third world countries like Nigeria, Afghanistan, and South Africa are suffering from worst poverty keeping forward all these conditions edible vaccines will prove a boon to overcome the disease and death rates. In Pakistan GMO food is being consumed but the labeling law is absent, whereas, equity, consistency, and fairness is the basic human right [7,8] maize, canola, cotton, and soybean are genetically engineered crops being used in different countries [9]. Other plants which are genetically engineered include poplar plant, gaining lots of interest [10]. Most recent development in biotechnology and bioengineering allows researchers to develop plant-based vaccines, plants are genetically manipulated [11] containing antigens to generate active immunity are called edible or green vaccines [12]. Plant-based vaccines are recombinant subunit vaccine, firstly the antigen to be expressed as capsid proteins are selected and these proteins are then expressed in plant tissues [13]. Production of pharmaceuticals in plants or animals is called biopharming, pharmaceuticals like antibodies, antigens, enzymes, hormones, anti-disease agents (e.g. Interferon and lactoferrin) and structural proteins (collagen) are produced in plants [14]. Now a day's vaccines, therapeutics, and proteins are being produced depending on the bacterial (*E. coli*) [15] mammalian and yeast cell expression system need high-cost purification, cold storage and are very expensive for patients to purchase [16] recent idea for oral delivery of plant-based vaccines therapeutic proteins and autoantigens this technology eliminates the need of cold storage, transportation, attenuated or inactivated pathogens, purification and sterile injections [17,18]. Genetically engineered crops are being adopted with the faster rate in developing countries [19] for this synthetic genetic circuits are used which may allow traits to be designed order in the future. Leaf and seed expression systems are used for proteins production latter is more suitable as it offers better storage for a long time other than these tows another way is suspension culture by using bioreactors for large-scale production, but the yield is compromised. cost of mammalian cell cultures, and all of this depends upon the product yield [20]. The principle advantage of transgenic plants for the atomic cultivating is generally minimal effort of vast scale generation, it is normal that recombinant proteins in plants might be delivered at 2-10% of the cost of microbial maturation frameworks and in addition 0.1% of the gene to protein duration for transgenic plants take up the preparation of expression construct, transformation, regeneration as well as the production and testing of numerous generations of plants, the testing part is essential to make sure transgene and its expression

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stability as well as biochemical activity of the product and the absence of undesirable phenotypic changes in the host plant, these processes will be done within two years depends upon the plant species [21]. Freeze dried plant cells expressing biopharmaceuticals and antigens are shielded in the stomach from acids and enzymes; cell wall of plants and are further protected by their accumulation in the cellular and subcellular compartments, like plastids and seed storage organelles in this way proteins remain protected from, chemical and enzymatic degradation [22] and harsh conditions in the gastrointestinal tract and reach the mucosal surface [23]. When the plant cell wall is digested by microbes present in the gut these antigens and biopharmaceuticals released to immune and blood circulatory system [17,24] these freeze-dried vaccines are subunit vaccines and their stability is desired property at room temperature, for they are consumed orally [25]. Edible vaccines can be used for the treatment of the number of gastrointestinal diseases in infants and children [26]. Lyophilized cells facilitate long-term storage [18,27]. The edible parts of fruiting plants containing vaccine are also very fascinating, as fruit or salad can be consumed raw without the loss of vaccine properties [12] in future, it will be easier to get vaccinated through genetically modified food (Figure 1). These vaccines will be preferable for developing countries where conventional medical care is not easily available to the common public, as it removes the need of a medical man and sterile injections [28]. these vaccines are created by familiarizing the desired gene into the DNA of selected fruiting plant, as a plant grows desired protein is produced [29]. The number of researchers is working on this concept for more than twenty years, vast research is performed on hepatitis B vaccine also the variety of plant species have been used to express antigen (like banana as can be consumed raw, is a leading candidate for edible vaccines, at a same time it is all time favorite of children. Potato is drought resistant and largely cultivated likewise, strawberry, apple, maize, tomato [30] are studied in number of beneficial researches for edible vaccine development most prominent studies include the development of plant-based vaccines for HCV, HBV [31] Influenza vaccines [32] respiratory syndrome virus and HIV vaccines [33]. Parenteral delivery of plant-based vaccines is well known and verified requires purification before delivery of antigens [34].

Automated Production in Plant Factories

Plant-based production of pharmaceuticals is inexpensive, as the presence of chaperones and protein disulfide isomerases allows folding and assembly of complex proteins [35]. A fully automated plant factory for the growth of tobacco plant is an example, where tobacco plants are cultivated in automated factory and agrobacteria having lofty copy of vector are introduced into the plant leaves, by automated vacuum infiltration technique, plants are harvested ground and the proteins are extracted and purified [36]. For plants based recombinant protein production current good manufacturing practices (CGMP) factory which is fully automated works for the production of biopharmaceuticals Table 1. Closed Plant Production System CPES, is a plant factory with artificial lighting is preferable to produce pharmaceuticals it allows stable plant production in controlled environmental conditions like light, temperature, humidity, and gas ensure efficient use of carbon dioxide gas and water.

Advantages

It is less dominant infrastructure, low operating costs, no need of bioreactors, aseptic liquid handling technologies production multiple products simultaneously, reduce production cycles from month to days, the plants are grown hydroponically in a rigid tray, the tray can be manipulated by automated machinery. Plant biopharming has four main production steps:

- Seeding
- Growth
- Infiltration
- Harvesting

Robotic Transport in Plant Factories

Robots carry empty trays to seeding module and transported to the growth module in growth module seeds are automatically watered for several and transferred to infiltration module where viral vectors are inserted to the plants and after this plants are allowed to grow further to facilitate the production of proteins, lastly trays are moved to the harvesting module and chopped into small pieces after extraction and purification, this enables cost-effective safe and fast vaccine production. Fraunhofer center for biotechnology research select tobacco plant as expression system because they multiply and maintain virus vectors well, the process begins and a robot picks up a tray containing plants, submerging the head of plants first in to the liquid by turning plants upside down, liquid contains water with vectors containing gene of interest the process is called infiltration [37] then plants are sent back to the growth module, the protein needed is produced within 1 week, after which the plants are harvested, leaves are cut down into small pieces and protein of interest is extracted and purified (Figure 2) [38,39].

Selection of host system

In *E. coli* in prokaryote [40] and Chinese hamster ovary cells [41] Pichia pastoris [42], Saccharomyces cerevisiae in eukaryotes expression

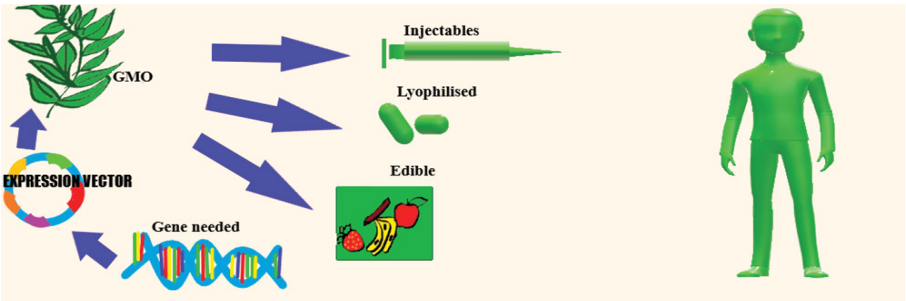

Figure 1: Genetically modified plant containing the gene of interest will produce protein needed, proteins are extracted and purified can be used as injected through parental route, plant leaves are freeze-dried and taken as tablets through oral route as oral vaccines, the edible parts like fruits can be consumed raw to develop immunity [28].

Company	Products	Focus	Pipeline	Status
Siphon	Monoclonal antibodies	Oncology & autoimmune diseases	Anti-HER2ADC	Phase- 3
			Trastuzumab	Market authorization
Mapp Biopharmaceuticals	Plant-based Therapeutics	Ebola therapy HIV	ZMapp	Phase-3
			MB66	Phase-1 completed
Ventria Bioscience	Plant based recombinant proteins & vaccines	Zoonotic diseases	VEN150	Phase-2completed
			VEN120	Phase-1completed
Greenovation	Moss based Therapeutics	Orphan diseases	Moss-AGAL	Phase-1
			Moss-FH	Preclinical trails
			Moss-GAA	Preclinical trails
Protalix	Plant-based enzymes	Cystic fibrosis, Fabry disease, immune &inflammatory diseases	PRX-102	Phase-3
			PRX-110	Phase-2
			OPRX-106	Phase-2
Medicago	Plant-based vaccines & enzymes	Influenza &Ebola	VLP Quadrivalent influenza vaccine	Phase-3
Icon Genetics	Plant-based vaccines & enzymes	Viral & bacterial diseases	Norovirus vaccine	Under development
Plant Biotechnology	Plant-based antibodies & therapeutics	MERS coronavirus & anthrax	DPP4-Fc	Phase-2 completed

Table 1: Showing different biotechnology companies their products, orientation, and pipeline status.

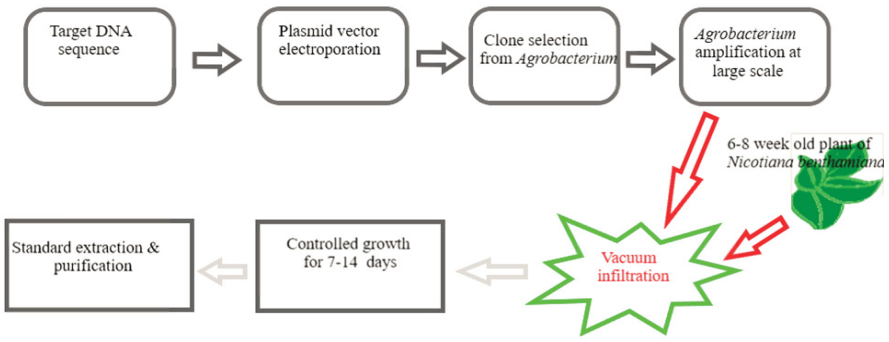

Figure 2: Process of biopharming followed by plant factories [79].

host system has been used for production of biopharmaceuticals, yeast is also a biotechnological host for the production of biopharmaceuticals [43-45] large-scale production of recombinant proteins depends upon microorganisms used to be the best suitable host, proteins expressed in mammalian cells [46] have similar properties as the natural origin, but still it is the less suitable for expression of the therapeutic proteins because of the inability of large-scale production and highly expensive culture cost [47]. Microalgae are being used as a production system for the production of vaccines and biopharmaceuticals [48,49] other new approaches are raised by advancing and rewriting the DNA of host [50] CRISPR has 9 technology can be used for creation off GMOs without the introduction of foreign gene, no doubt gene editing is a powerful technology [51-53]. Plant biotechnology is a joint venture of tissue culture and genetic engineering genetically modified organisms are products with altered genetic material to get desired protein utilizing recombinant technology plant part which is Utilize should be well studied and allow effective risk assessment of the transgene of interest. selection of host system is important for efficient protein production.

- Use self-pollinating plants [54]
- Use leafy plants for huge biomass production
- Use of cereals and leguminous plants
- Tissue that can be consumed raw and should not produce toxic molecules [55]

Plants offer following advantages for the recombinant protein production

- The lower production cost
- High scale production
- The ability to correct folding
- Post-translational modifications of the expressed proteins
- Minor differences in glycosylation pattern [56]
- Safe articulation frameworks as the plant cells are free from potential human pathogens or prions, oncogenic DNA arrangements and endotoxins.

Plant cells are most suitable for the production of biopharmaceuticals offering the most economical production with simplicity, post-transcriptional modifications and they are free of animal pathogens [57]. Tobacco has huge biomass production capacity. Phenolic compounds may release during an extraction course that might be detrimental to the downstream processing by expression of proteins from leafy crops and this could be unstable green leaf host system is easy to engineer, it can lead to problems if the protein interferes, with plant development and also purification is difficult due to the presence of pigments, alkaloids, and polyphenols [58-60] high protease activity

and low protein content is another disadvantage of leaf system [61,62]. Tobacco plant have great ability to produce a large amount of green leaf per acre and the Agrobacterium-mediated transformation is successful in tobacco but high amount of nicotine is undesirable, leafy plants have advantage of producing biomass, recent study tobacco is used as an expression system production of immunogens against Herpes Simplex Type 2 [63] but have limited shelf life and unstable protein expression on the other hand proteins stay stable in seeds at room temperature tobacco is being used Seed is main storage organ and helps in downstream processing, avoiding the recombinant protein by phenolic compounds and prevent degradation. Seed is beneficial over the systems as they accrue vast amounts of protein in a comparatively small volume and it provides an environment that may endorse protein accumulation as well as it inhibits degradation that may consequently provide long-term storage [64]. Example of this is antibodies that may present at prominent levels in seeds and stable with no loss of activity [65]. Seeds lack compounds such as phenolic and alkaloids that may present in leaves and obstruct with downstream processing by means of fouling membranes as well as chromatography media. The particular organelles in the seeds present additional advantages such as improved stability and ability for accumulation, bio encapsulation, and enhanced processing approaches for economic purification [66]. Seeds are preferred as they are easily transportable and have enough storage planet biotechnology is one of the leading pharmaceutical companies uses transient and stably transformed tobacco plants for the production of pharmaceuticals and get high levels of expression between 0.5 to 3 grams/Kg [67] another company named as Wuhan health gen biotechnology better known as Cryogen uses rice as a host endosperm cell are taken and used to express recombinant proteins [68] likewise seemed is another biotechnology company, uses soybeans as host system for the production of seed-based pharmaceuticals, transgenic soybeans contain 40% by weight of desired protein [69]. Hairy roots of plants can be used as good mean of producing biopharmaceuticals, is also safe and inexpensive and have been used for production of many recombinant proteins, the proteins expressed in hairy roots are released in to the medium, product purification and homogeneity are the clear benefits of root system, for example hairy roots of tomato were used for the expression of rabies glycoproteins ricin toxin B chain antigen [70]. Every system has its advantages and disadvantages a perfect expression system with all economic features but efficient transformation and regeneration could be helpful to get a better expression system.

Plant transformation types

There are two main types of transformations, the stable transformation which includes nuclear and chloroplast transformations and second, is transient transformation which includes plant virus-based system.

Nuclear transformation: This transformation involves the integration of foreign gene in the genome of a plant. This may be done either by Agrobacterium-mediated transformation of dicotyledonous or monocot plants by gene-gun method, as leaves of dicots, seeds of dicots and monocots can be utilized for nuclear transformation [71,72] such transformations resulted in genetic expression of recombinant protein gives most stable expression [73,74]. Nuclear transformation allows to target proteins to various subcellular regions like endoplasmic reticulum, plastids, vacuole, apoplast, which facilitate correct post-translational modification (Figure 3).

Chloroplast transformation: This method used to transform chloroplasts by using a gene gun to integrate the transgene into the chloroplast genome [75]. Chloroplast transformation does not give post-translational modifications such as glycosylation [76] for production of heterologous proteins, which does not need the post-translational modification, high copy number, no gene silencing, multiple genes can be expressed like in operon [73] and integration of gene of interest by homologous recombination [77,78].

Transient expression: Transient expression system utilizes the beneficial characteristics of plant pathogens to infect plants [79] to avoid the escape of pharmaceutical-producing plants to the wild environment we use transient expression strategy, in transient expression, the foreign genetic material does not integrate into the genome of the plant, this could be done via agro-infiltration, viral vectors or by gene gun. Several of binary vectors called pEAQ vectors for transient expression developed that gives elevated level of transgene expression in very short time [80] secondly, inducible gene promoters are used gene remains inactive and will only activate when sprayed by chemical inducer after harvesting it proves effective as far crop is not damaged by wind or herbivores. However, the protein yield is lower.

Plant viral system: Expression of recombinant protein done by a viral vector is a method for examining protein and its desired character in a plant. Virus infected plants are used to get antigens and antibodies with rapid onset of expression and more than one vector can be used in the same plant. Dicots leaves are most commonly used like of tobacco. The plant produces the high quantity of desired protein within 1-4 weeks of inoculation [81]. Virus-based system consists of two main systems, epitope presenting system, with fused antigens to coat

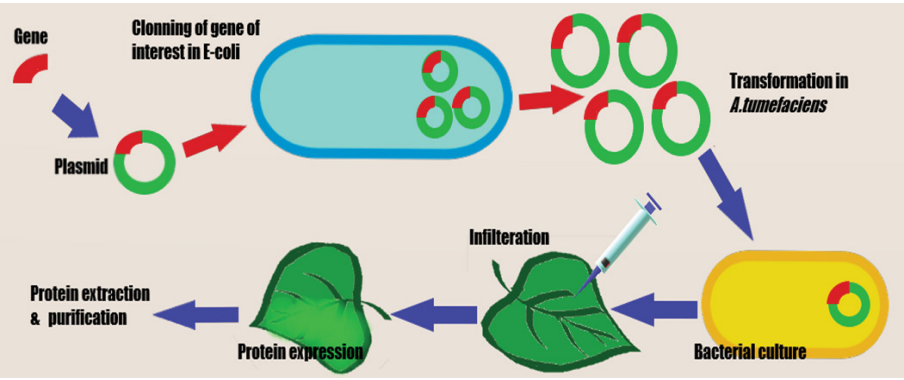

Figure 3: The gene of interest is introduced into the plasmid and cloned in *E. coli* after cloning is plasmids are transformed in *A. tumefaciens* the bacterial cell in cultures and then agroinfiltration is done and expressed proteins are extracted and purified [83].

proteins and second, is polypeptide expression system, with unfused recombinant proteins which are accumulated by plant but require additional concerns in term of containment [82].

Gene Gun Mediated Transformation in Biopharming

It is known as Particle Bombardment Biolistic Microprojectile siege Particle increasing speed.

Particle inflow gene gun

Utilizing a quality weapon specifically shoots a bit of DNA into the beneficiary plant tissue, tungsten or gold dots are covered in the quality of intrigue and discharged through a ceasing screen, quickened by Helium, into the plant tissue, the particles go through the plant cells, leaving the DNA inside, DNA-covered micro carriers are stacked on micro carrier, miniaturized scale transporters are shot towards target tissues amid helium gas decompression, a halting screen set permitting the covered micro projectiles to go through and achieve the objective cells [83-85].

Helios gene gun system

The helium beat clears the DNA-or RNA-covered gold micro carriers from within mass of the example cartridge, the smaller scale bearers quicken for most extreme entrance as they travel through the barrel, while the helium beat diffuses outward, the spacer keeps up the ideal target separate for *in vivo* applications and vents the helium gas from the objective to limit cell surface effect (Figure 4) [86,87].

dvantage

- This method can be used to transform all plant species.
- No binary vector is required.
- Transformation protocol is relatively simple.

Disadvantages

- Difficulty in obtaining single copy transgenic events.
- Excessive cost of the equipment and micro-carriers.
- Intracellular target is irregular (cytoplasm, core, vacuole, plastid, and so forth).
- Transfer DNA is not protected.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens is a bacterium that infects plants and causes crown gall disease, because of its ability to transfer the segment of DNA it is used for the production of transgenic plants, researchers have used it by inserting the DNA of interest between the T-DNA of *Agrobacterium*, after this crown gall disease will not occur as the plasmid is constructed to disarm tumor-inducing characteristics (Figure 5) [88]. Agrobacterium tumefacient-mediated transformation involves the transfer of any DNA located between 25 bp direct repeats present at left and right borders that delimit the single strand T-DNA, is transferred in to the nucleus of plant cell, and integrates into the plant chromosome by illegitimate recombination [89,90] however all of T DNA copies do not integrate but transcribed resulting in transient expression of foreign gene [91] transient expression yields higher protein levels than stable one, and also not affected by position effects [92] transient expression declines sharply after 60-72 hours, as a result, a result of post-transcriptional gene silencing [93]. *Agrobacterium strain* GV3103 is used for transient expression [94].

Recombinant DNA stability in plant

Inserting recombinant DNA inside the plant genome may undergo inactivation and prevents its expression inside the plant cell. This DNA inactivation occurs due to the presence of repeated homologous sequences, co-suppression and recombinant DNA methylation [95]. These issues can be overcome by following:

- To incorporated numerous duplicates of DNA
- Overexpression of duplicates of DNA
- Selecting lines with a solitary addition of the transgene
- Not utilizing tedious homologous arrangements
- Selecting stable recombinant lines
- Creating site-particular recombination frameworks

The post-translational alteration is in charge of

- Proper organic action
- Stability and collapsing of the recombinant protein
- Many proteins to amass into the dynamic multimeric frame require complex post-translational adjustment steps.

Glycosylation plays a key role for many physiological activities of a glycoprotein is one of the critical post-translation modifications [96]. Phosphorylation is the key factor for the recombinant enzymes which are only activated after phosphorylation. Tyrosine phosphorylation is used by animals, while both the serine-threonine phosphorylation and tyrosine kinase pathway are active in plants.

Optimization of foreign gene sequences for expression in plants

Yields of any biopharming product are not predictable because

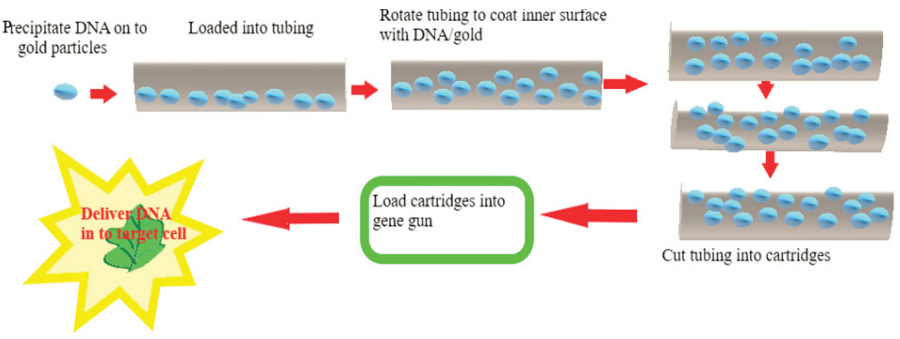

Figure 4: Helios Gene gun.

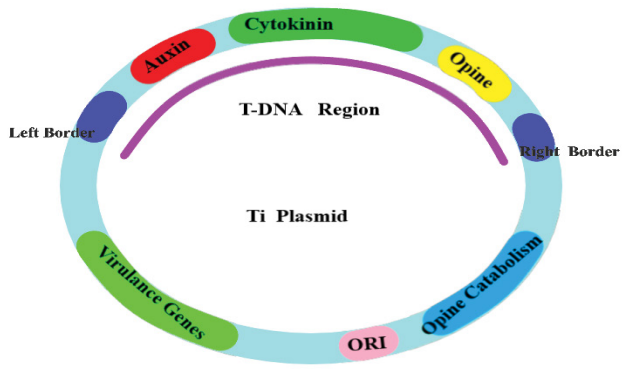

Figure 5: Plasmid construction for Agrobacterium-mediated transformation.

it depends on the number of intrinsic properties of proteins, the host and the production strategy. Plants have different codons, yet foreign DNA may optimize for expression in plants to enhance translation and enhance protein yields, expression may be enhanced via, the use of tissue-specific promoters [97] improving transcript stability and viral sequences used for translational enhancement [98]. Protein expression may be enhanced via the use of introns in the recombinant DNA molecule, the suitable promoter selection is a key factor for protein expression by enhancing transcription of foreign gene by that promoter, the foreign gene expression can be targeted to specific tissues of lower metabolic activity like endosperm by using tissue-specific promoter, promoter with wound inducible defense gene are best reported for overexpression of recombinant proteins in plants. Co-expression of host chaperons along with transgene expression is another technique to improve expression, host chaperons gathering could be invoked by heat, membrane fluidizers, and osmolytes, plasmid-derived overexpression may not need [99].

Downstream processing

Downstream processing usually refers to the recovery and purification of the recombinant protein from plants leaves. The production cost depends on a protein expressed, biomass produced and production scale, greater the biomass greater the processing cost [100]. Processing of leaves necessitate interest, leaves should be processed instantly after harvesting or must be stored to avoid degradation of proteins by proteases. Affinity tags must be used to facilitate protein recovery. These tags then removed after purification to restore the structure of the purified protein from its inhabitant situation. Problems meet at the time of protein extraction mainly includes proteolytic degradation and structural modification, because of the reaction with phenolic compounds [101]. Purification with quantity and quality are challenging for seed system, Purification strategies rely on oleosin fusions in oil bodies or maybe polymer fusions or other sections can develop any suitable process for the isolation of proteins [102]. Approximately more than 80% of the entire production, cost of recombinant protein is linked with the downstream processing. A plant-based system is beneficial as many of the recombinant proteins can be used as part or unprocessed material reducing the downstream cost. As it's important to increase the production and recovery of the target proteins, endoplasmic reticulum may be essential for the processing, disulfide bond formation, assembly and glycosylation of proteins. Rhizosecretion of recombinant protein in the hydroponic medium in roots is an approach that may help in simplifying the downstream processing and may increase the protein yield.

Conclusion

Biopharming is recent technology serving in production of various pharmaceutical products which are already being produced in many pharmaceutical companies by bioreactors using algae, bacteria and mammalian cell culture, where production is followed by expensive downstream processing, but in case of plants production is quite suitable and affordable, however challenges remained partially meted in the selection of suitable host system, transformation technology for the production of desired proteins, recombinant gene stability and optimized expression, all of these play their equal role for the attainability of final product, until now biopharmaceuticals are being produced so well and being consumed also but still promising techniques are required to get most accurate, stable and cost-effective expression.

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