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Plant molecular farming: Production of metallic nanoparticles and therapeutic proteins using green factories

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View Article Online
DOI: 10.1039/C9GC00335E

CONTENTS

1. Introduction

2. Phyto-nanotechnology and plant-made nanostructures

3. Living plants in nanoparticle synthesis: current status and future prospects

    Endogenous NPs biosynthesis using living plants

    Exogenous NPs biosynthesis using living plants

    Mechanistic aspects: Responses of plant proteins to heavy metal ions

4. Plant molecular farming: production of therapeutic proteins

    Production of collagen

    Production of gelatin

    Production of elastin

    Production of recombinant anti-cancer monoclonal antibodies

    Production of recombinant anti-cancer vaccines

5. Challenges and opportunities

7. Conclusion

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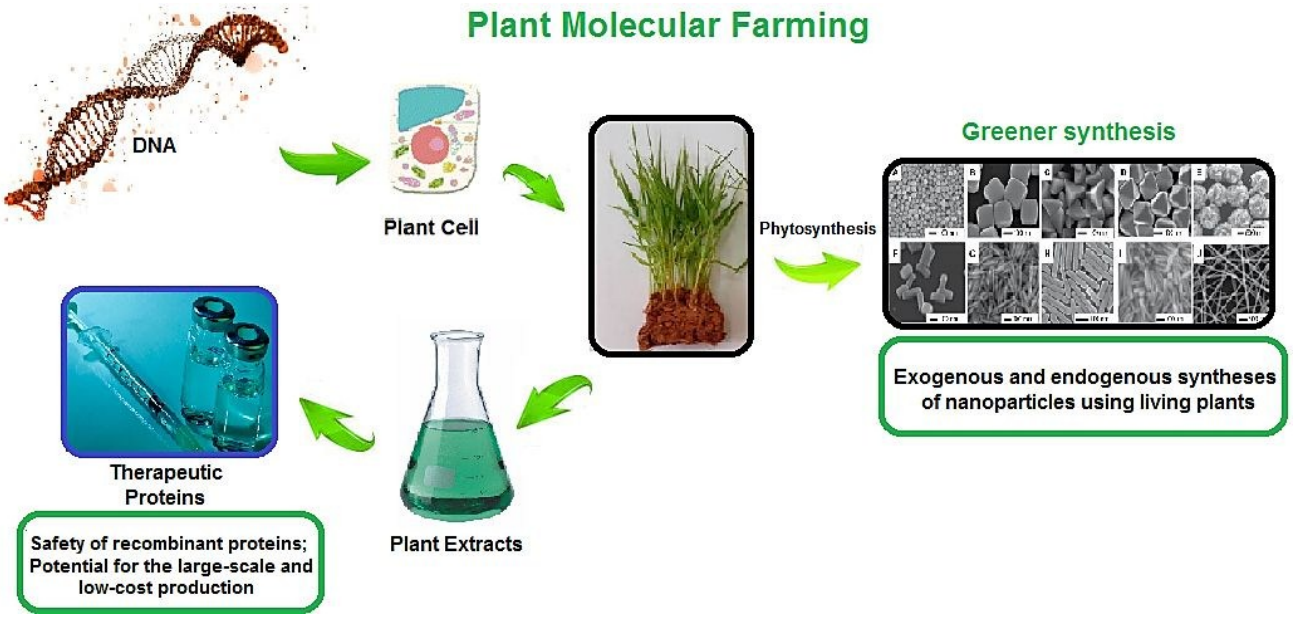
Graphical abstract: Exogenous and endogenous syntheses of nanoparticles using living plants, and the plant nano-molecular farming of proteins namely collagen, gelatin, elastin, recombinant anti-cancer monoclonal antibodies and recombinant anti-cancer vaccines, are discussed.



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Abstract

Plants have numerous biological, clinical, pharmaceutical and medicinal purposes for many years; however, their use as a general platform for preparation of desired pharmaceutical and biomedical is relatively current. Secondary metabolites with remarkable and diverse biological functions are produced by medicinal plants. Significant advancements in nanosciences have enabled various applications in the development of new generation of drug molecules. Due to the application of toxic solvents and high energy consumption of conventional physical and chemical approaches, greener and eco-friendly methods are essential and vital. Plants can provide an outstanding alternative for the production of phytomaterials and biomaterials, and this review highlights the exogenous and endogenous syntheses of nanoparticles using living plants. Additionally, the plant nano-molecular farming of proteins including collagen, gelatin, elastin, recombinant anti-cancer monoclonal antibodies and recombinant anti-cancer vaccines, are discussed.

Keywords: Plant molecular farming; Living plants; Nanoparticle synthesis; Greener methods; Proteins; Recombinant anti-cancer monoclonal antibodies; Recombinant anti-cancer vaccines

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1. Introduction

Plants have been used for biomedical, pharmaceutical and medicinal purposes for centuries; however, their use as bio-factories for the production of pharmaceuticals and valuable compounds is relatively recent. In fact, the production of biomaterials should be performed in systems which can provide high yield and affordable down-processing. Plants offer many advantages over mammalian and insect cells, because the system is fully scalable, cost-effective and avoids possible contamination with mammalian pathogens. Manipulated plants can be applied for the production of compounds including chemicals for the generation of biomaterials, although they do not have the diversity of polymers synthesized by current chemical polymerization techniques. Nevertheless, by deploying technology of gene-transfer, it is feasible to define the compositions of the compounds of interest and have, in general, a control over its physicochemical properties and functionality, which is hard to achieve, using chemical techniques.[1]

The field of biotechnology and gene-transfer techniques has experienced considerable advances. Originally, the production of valuable pharmaceutical compounds and antibodies was performed using transgenic plants, but because of negative public perception, in addition to the potential risk of gene escape, the full implementation of this technology was constrained. It is for this reason modern systems deploy transient expression of heterologous proteins that relies on viral vectors, which do not result in transgenic plants. Plants are excellent resource in greener production of biomaterials and in this critical review the recent progress on plant-based fabrication of biomaterials such as collagen, gelatin, polyhydroxyalkanoates (PHAs), silk and elastin, has been highlighted.[2-5]

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The application of phytomedicines has increased due to their therapeutic values when compared to allopathic medicines as these bio-compounds exhibit fewer side effects. A better understanding of phytopharmaceutics function and kinetics should help in designing novel drug molecules and effective treatments.[6]

Plant extracts contain important secondary metabolites including alkaloids, terpenoids, phenolic acids and flavonoids which are the key compounds participating in the preparation of bulk metallic nanomaterials and nanoparticles (NPs).[7] Such metabolites are routinely participating in the redox reaction to synthesize eco-friendly NPs. It is well known that various plants, herbs and spices are the key sources of powerful antioxidants as phytochemical subunits in leaves, stems, seeds and fruits.[8,9] The utilization of plant-based NPs and other nanoparticle embedded by-products is very important as it brings forth a crucial symbiotic association between plant science and nanotechnology. This kind of correlation offers an inherently greener approach towards nanotechnology, often referred to as green nanotechnology.[10,11] One of the major roles of nanotechnology is drug delivery in which small particle size leads to accessibility of whole surface area of the drugs and in turn enables rapid dissolution in blood. In addition, drug delivery is targeted in a specific manner to a desired site of action. Due to its very minute size, microspheres and liposomes can easily pass via sinusoidal spaces in bone marrow and spleen as compared to the other system. NPs increase the consistency of proteins against enzymatic degradation and exhibit superiority over traditional methods in terms of efficiency and effectiveness.[12]

Drugs or other active compounds can be loaded on engineered NPs for effective targeted delivery to specific sites in an organism. Notable efforts have been made to examine the broad applications of engineered NPs within human systems, mainly for targeted delivery of drug,

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cancer therapy and various genetic disorders which can be well addressed by their effective utilization.[5] Recently, Aminianfar *et al.,*[13] observed that botulinum toxin type A toxicity has been diminished, when it coupled with nano-silver (Ag) and intraperitoneally injected into rat.

Phytomedicines have become more popular for their potential usage to cure many kinds of ailments with high therapeutic values and less toxicity. However, there are some limitations which hinder the proper application of phytomedicines; such barriers can be overcome by incorporating nanosciences to develop effective drug delivery systems. It is possible to minimize the size of phytomedicine by modifying surface properties, aqueous solubility and permeability via biological membranes. Innovative drug delivery systems including nanospheres, phytosomes, liposomes and niosomes have been noted for their effective ability for site-specific delivery. Incorporation of such herbal delivery systems helps enhance the stability, increase in solubility, development of pharmacological activity, and sustain delivery, improvement in macrophage distribution and protection from chemical and physical damages.[14]

An innovative phyto-nanomedicine prepared via well-formulated routes of synthesis, by virtue of their size, increases dissolution and bioavailability of drugs while reducing its dose. *Cuscuta chinensis,* containing lignin and flavonoids as active therapeutic compounds, shows poor aqueous stability and solubility upon oral administration. The nano-sized drugs from *C. chinensis* were produced by nano-suspension techniques for antioxidant and hepatoprotective effects. Similarly, *Radix salvia* NPs have been synthesized by spray drying method for coronary heart disease and myocardial infarction.[15,16] Potential utilization of nanotechnology techniques leads to increased bioactivity and bioavailability of phytomedicine by minimizing the size of particles, surface modification, entrapping the phytomedicine with various polymers of nano materials. In future, it is necessary to focus on designing and development of new

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multifunctional novel nanomaterials and *in vivo* studies of their formulations for effective application in the pharmacological fields. [15,16]

2. Phyto-nanotechnology and plant-made nanostructures

Relative to traditional methods of nanoparticle synthesis using toxic and hazardous materials, plant-based eco-friendly and greener nano-approaches for the assembly of NPs are showing major advantages. Plant extracts are renewable in nature and often are processed in eco-friendly aqueous medium. Moreover, reaction conditions used in production processes are mild. [15-19] Additionally, plant extracts and phyto-nanoproducts are receiving consideration as they cost effective, non-hazardous and energy efficient. Based on green chemistry principles, three main steps should contribute in the production of greener NPs, such as i) selection of biocompatible and non-toxic solvent medium, ii) environmentally favourable reducing agents, and iii) nontoxic substances for stabilization of the ensuing NPs. [20,21]

There are relatively fewer studies in phyto-nanotechnology arena because of the complexity of plant systems and other reproducibility limitations. Phyto-nanotechnology has high potential in the production of different NPs employing the extracts of different parts of plants such as leaves, seeds, flowers and roots.[1, 22] Such synthesized biological nanomaterials have notable applications in medicine, mainly in preparation of novel pharmaceuticals, imaging, drug delivery, diagnosis methods and in making effective nano devices.[23] Hence, greener production of NPs is the key building block for developing new therapies to control various epidemic diseases.[24]

The fast growth in the commercial applications of nanomaterials is leading to an intensive search for greener routes to prepare NPs; particles in nanometers size i.e. 10^{-9} m.[25]

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Developing alternative eco-friendly methods for generating NPs is imperative.[26] During the last decades, scientists directed their investigations to the biosynthesis of nanomaterials as a 'bottom-up' track; numerous organisms could synthesis NPs under ambient environment (pressure and temperature) avoiding the generation of dangerous agents and hazardous by-products.[27] Historically, the biosynthesis of NPs using plants was reported in the early 1900s and so was the accumulation of colloidal Ag in organs of living organisms [28] and bioreduction of ions by plant roots.[29] Also, the preparation of metallic NPs using plant seed extracts has been documented,[30] the reduction of Ag nitrate;[31] including inside the plant cells.[32] Although a change in color of Ag nitrate into yellow[30] or yellowish-brown[29] was observed as an indicator for nano-Ag formation,[33] the ensuing reduction products were not characteristically analyzed.[29, 30] Notably, the well-defined NPs preparation by a ground plant biomass and their characterization was demonstrated using alfalfa plant, *Medicago sativa* [34] with experimental proof of synthesis in a living vascular plants.[35] Several reports have shown potential applications of various plant species to generate NPs including leaves [36, 37], seeds [38], flowers [39], fruits [40], latex [41], tuber [42], bark [43] and cultured tissues.[44] However, few articles examined the capability of live plants to generate NPs.

3. Living plants in nanoparticle synthesis: Current status and future prospects

The main puzzle in the phytosynthesis of NPs is about the origin of this phenomenon as the procedure in living plants has not been entirely elucidated.[45] One school of thought is that NPs can be prepared endogenously; within their cells such as *Arabidopsis thaliana*,[46] *Brassica juncea*,[47-51] *Festuca rubra*,[50] *M. sativa*,[35, 47, 48, 50, 52] and *Sesbania drummondii*.[53] The exogenous synthesis of NPs using the whole plant has been another approach [54-59]. In fact, it was revealed that plants have excellent capability to accumulate heavy metals thus accomplishing

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detoxification;[60] several such studies have demonstrated the hyper-accumulation and detoxification of heavy metals using plants, such as *Arabidopsis halleri* and *Thlaspi caerulescens*.[61,62] Diverse genres of plants such as *Acanthopanax sciadophylloides*, *Maytenus founieri*, *Brassica juncea*, *Ilex crenata*, *Sesbania drummondii*, and *Clethra barbinervis* have displayed capability for phytoremediation of heavy metals.[60-63] Metalloids and heavy metals are significant environmental contaminants, and are harmful and hazardous at very low concentrations. Biosorption of metals from aqueous solutions using plant biomass has garnered consideration because it has revealed promise for the removal of contaminants and pollutants from effluents in an eco-friendlier manner. The tolerance of heavy metals by plants has interested scientific investigators to investigate the related biological mechanistic aspects, genetics and physiology of metal tolerance in hyper-accumulator plants.[60-63]

3.1. Endogenous NPs biosynthesis using living plants

The endogenous biosynthesis of NPs by plants depended mainly on the ability of these living organisms to use their roots to extract metals from the medium they grow in. Such hyper-accumulators plants have the capability to accumulate meaningful metal concentrations in their cell wall, vacuole, and the cytosol. The purpose of this procedure was to make them nontoxic and retain them at a distant place from active metabolic sites in plant cells. Plants such as *M. sativa* [35, 52] and *B. juncea* [49,51] grown on metal rich solid systems, such as agar [35,52] and soil,[49,51] confirm their capability to biosynthesize NPs inside their tissues.[35, 49, 51-53] Briefly, when seedlings of *M. sativa* grown on potassium tetrachloroaurate, $KAuCl_4$,[35] and $AgNO_3$,[52] in rich agar system, i.e. Au^{+3} and Ag^{+1} wealthy sources, Au NPs and Ag NPs were formed inside their living tissues.[35,52] The dispersion of Au^0 via the plants in their roots and the shoots suggested that

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they actively transport Au atoms; existence of Au NPs in a size range 2 to 20 nm indicated nucleation of the particles inside the plants in favored zones. Furthermore, the NPs consisted of Au^0 with no oxidized Au present in the FCC configuration. They were multi-twinned and the lattice parameter calculated was 0.23; the approximate spacing between the 111 planes. The formation of Au in a low-energy configuration state for Au^0, an icosahedral structure; has suggested the slow reduction rate or the equilibrium conditions.[35] In addition, *M. sativa* roots were capable of accumulating Ag as Ag^0 from the agar medium and then transferring it to the shoot in the same oxidation state; absorbed Ag^0 undergo nucleation and NPs were formed as an associated step. Nanostructures were dispersed throughout the plant in small assemblies like nanowires with sizes averaging from 2 to 20 nm in diameter.[52] Also, Au NPs of a 5-50-nm diameter have been synthesized within *B. juncea* tissues grown on soil supplemented by $AuCl_4$, where the plant contained around equal amount of Au in the metallic and oxidized states; only half of the absorbed Au by the plant was reduced to the metallic state.[51] A mixture of NPs containing Au, Ag and Cu was synthesized by the seedlings of the same plant grown on metal abundant soil.[49] Furthermore, using complexing agents such as thiocyanate enhanced the Au^0 uptake from Au-enriched media.[64] Pure metal NPs including Ag, gold (Au), titanium, chromium, zinc, and cobalt have been synthesized deploying the plants metabolic pathways and specifically, the possible reduction of the above-mentioned metals using *M. sativa* and *Ipomoea lacunosa* have been studied followed by deposition of the particles on supports of steel.[65] Liquid culture was selected over a soil-based system to accurately control the dosing of metals in this hydroponic method that allowed the clean washing of plant material from the growth substrate to reduce contamination with non-plant material.[46] Thus, plant-mediated Au NPs were synthesized hydroponically using seedlings of *S. drummondii* intracellularly grown in aq. $KAuCl_4$.

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DOI: 10.1039/C9GC00335E

Apparently, the roots of this plant trap ions from solution because of the interaction of Au^{+3} with the carboxylic acid moieties of the cell wall where the reduction of metal ions (MIs) occurred at the external boundary of the cell wall or the inner boundary of cytoplasmic membrane; the ensuing NPs were transported via the cytoplasm, symplastic pathway, to the shoots. Various spherical Au NPs close to cell organelles were formed in a range of 6-20 nm sizes.[53] Furthermore, the hydroponically grown *M. sativa* and *B. juncea* seedlings can intracellularly generate Ag NPs,[50, 54, 66] Au NPs [47] and Pt NPs;[47] Ag NPs biosynthesized by both plants were stored in their tissues (about 50 nm) when seedlings have been exposed to aq. $AgNO_3$.[54] The exposure of *B. juncea* seedlings to either aq. $AgNO_3$ or aq. Ag $(NH_3)_2$, generated NPs in shoot and root systems and were found also in vascular bundle tissues and cell walls in smaller amounts. From the nitrate source, spherical NPs of size range of 5-140 nm in the leaves, 40-60 nm in the stem, and 10-30 nm in root. However, NPs prepared from ammonia complex were 5-50 nm in leaves.[66]

The *in vivo* generation of Ag NPs was noted in the leaves, stems and roots, of the *B. juncea, Festuca rubra and M. sativa* plants which were grown in Hoagland's solution before the exposure to aqueous $AgNO_3$. In roots, Ag NPs were located on the cell wall of the xylem vessels, in the cortical parenchymal cells, and in zones analogous to the pits. In leaf, Ag NPs of varying shapes and sizes were situated in the cytoplasm, close to the cell wall, and inside chloroplasts. Three plant species, however, did not have Ag NPs in the phloem. The contents of antioxidants and the reducing sugars, responsible for the fabrication of Ag NPs, are variable among the species increasing the improbability that only single substance was responsible for this reduction.[50]

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DOI: 10.1039/C9GC00335E

Additionally, Pt can be accumulated by the *B. juncea* and *M. sativa* roots via ion exchange at lower concentrations whereas at elevated concentrations, added absorption occurs via concentrated dependent and facilitated diffusions. The endogenous generation of Pt NPs with varying morphologies and sizes (from 3 to 100 nm) in the epidermal root cells was revealed. The presence of Pt in the root, accumulating the metal in the outer central vascular system, epidermal cells, and middle cortical cells has been shown and being mobile greater quantities were found in the cortex for transport to the shoots.[47] Once taken-up, the movement of Pt to the shoots was also influenced by the pH of the solution, as large quantities of metal transported at acidic pH; uptake apparently decreased with the increasing pH. Upon exposure to Pt^{+2} solutions in water, both species were able to translocate and accumulate Pt to their above ground portions; at all concentrations, the roots immobilized larger amounts of Pt than the shoots in both the species. The lower concentration of Pt in the shoots of *M. sativa* in comparison to the roots was attributed to the binding of Pt to the protein fractions of the root cell walls and/or pectin, leading to a reduced amount being accessible for transport to the above ground parts of the plant. In contrast, *B. juncea* roots were able to translocate more masses to their shoots than *M. sativa*.[47] Ag and Au salts were absorbed by *B. juncea* and transported in the plant either as metal nanoparticle or as salt. Furthermore, spherical NPs of Au^0 and Ag^0 were located in stems, leaves and roots. In the cell walls, they were found in the leaves with sizes of 2-100 nm; chloroplasts being the sites for the utmost reduction of metal salts to NPs due to the richness of reducing sugar. The endodermal layer and Casparian strip of the root were ineffective barriers to the uptake of Ag and Au metals as they were dispersed throughout cytoplasmic and vacuole compartments of the root, upper stem, lower stem, and leaves. Au and Ag NPs were reported in both the xylem and phloem of *B. juncea*. The presence of NPs in cell walls demonstrated apoplastic transport via the cell wall of

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these metals.[66] Moreover, liquid culture-grown seedlings of *A. thaliana* (L.) up on exposure to an aqueous solution of potassium tetrachloropalladate, K_2PdCl_4, resulted in the formation of well-dispersed, spherical Pd NPs with a mean diameter of 3 nm up to 32 nm. Pd NPs were mainly concentrated in the apoplast and wall areas of cell junctions in the leaf; the binding to sulfhydryl, amino and carboxyl groups were vital before bioreduction.[46]

3.2. Exogenous NPs biosynthesis using living plants

In general, the bio-reducer and bio-stabilizer entities are secreted in response to metal stress as a strategy for tolerance. One of the botanical mechanisms to withstand the metal stress involves the secretion of exogenous anti-oxidants[67] including carbohydrates [58,68] and phenolic compounds.[55,56,58,59] Numerous phenolic compounds are known to form stable complexes with widely distributed toxic metals. Polyphenolics, described by at least one aromatic ring (C6) with additional hydroxyl groups,[26] are a group of secondary metabolites of low molecular weight that provide heavy metal stress tolerance by chelating MIs [26,55,56,58,59] or by foraging heavy metal stress prompted reactive oxygen species (ROS).[69] Therefore exogenously, the phyto-generation of metallic NPs was based on the reducing capacity of plant.[56] The complicated redox control for physiological pathways in plants is restrained by an array of biomolecules such the reducing sugars, enzymes, polyphenols and organic acids.[55,56,58] During seed germination and early seedling growth, some seeds release phenolics. Interestingly, these phenolic compounds can reduce Au^{+3} of hydrogen tetrachloroaurate (aq. $HAuCl_4$) and promote generation of Au NPs owing to the electron donating capacity and this appears to be a model Au^{+3} tolerance mechanism shown by *Vigna unguiculata*, where toxic Au^{+3} was converted to less/non-toxic AuNPs;[59] seedlings of *V. unguiculata* generate mono-crystalline spherical Au NPs in a size range

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DOI: 10.1039/C9GC00335E

of 5-10 nm.[59] Similarly, hydroponically grown seedlings of *M. sativa* plant could reduce Ag^{+} into Ag^{0} and generate Ag NPs extracellularly, when they were exposed to aqueous solution of Ag nitrate (aq. $AgNO_3$).[58] Biosynthesized single crystalline Ag NPs had well-resolved lattice fringe patterns of 0.23 nm, the estimated spacing between the 111 planes. Raie and coworkers used the cultured tissue, callus, derived from hypocotyl, of the same plant to reduce ions of Ag into metal nano-form wherein the exposure of living seedling and callus to the aq. $AgNO_3$ put the biomass in a metal stress condition thus secreting different anti-oxidant exudates including carbohydrates, polyphenolics and proteins.[55,58] In the case of the bio-production by callus, Ag^{0} were poly-shaped including spherical, disk and irregular shapes,[55] however they were spherical in the case of seedlings.[58] Remarkably, the pH of aq. $AgNO_3$ played an important role in metal biosorption and nanoparticle morphology; mono-dispersed spherical shape NPs in a size range from 2 nm to 7.5 nm were bio-synthesized by seedling at pH 10.[58] Under the same alkaline condition; i.e. pH 10, the size of NPs which were bio-generated by callus ranged from 35 to 40 nm.[55] While, others have demonstrated that the root system of plants can effectively interact with harmful metal species via ligands located on cell surface and the cell wall. Hence, ions were reduced into metals due to the transmembrane dehydrogenases/reductases of root surface cell plasma membrane. These enzymes have a serious function in root surface-facilitated reduction routes by drawing electrons via the oxidation of NAD(P)H to NAD(P) and in turn can effectively reduce Ag^{+} to Ag^{0} and Au^{+3} to Au^{0} which nucleated to generate Ag NPs[56] and Au NPs,[57] respectively. Exogenously, the root system of hydroponically grown seedlings of *Amaranthus gracilis, Brassica juncea, Catharanthus roseous, Cannabis sativa, Cicer arietinum, Cynodon dactylon, Euphorbia hirta, Lycopersicon esculentum, Medicago sativa, Ocimum sanctum, Phyllanthus fraternus, Portulaca grandiflora, Tagetes erecta, Triticum aestivum, Vernonia*

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cinerea and *Vigna mungo* could generate Ag NPs and Au NPs. The exposure of seedlings to aq. $HAuCl_4$ [57] and aq. $AgNO_3$[56] caused the production of spherical NPs in a size range 5-50 nm.

3.3. Mechanistic aspects: Responses of plant proteins to heavy MIs

Basically, either exogenously or endogenously, the plant-mediated NPs synthesis is result of a redox reaction where the anti-oxidant activity of the phytochemicals plays an imperative role in metal reduction. The reduction potential of ions into metals in a common oxidation state for Au, Ag, Pt, Pd, and Cu are 1.0 V, 0.8 V, 0.74 V, 0.64 V, and 0.35 V, respectively according to standard hydrogen electrode series. Hence, a swift ion reduction and nucleation of metallic seeds are promoted via bio-molecules as a starting by induction phase. Spontaneously, such small, reactive and unstable crystals accumulate into large aggregates; i.e. growth phase. Finally, the shapes and sizes of the aggregates are rendered energetically favorable and some biomolecules act as stabilizing agents for the NPs.[70]

Generally, metallophytes or hyper-accumulators can uptake great amounts of heavy metals in the soil, and can tolerate high levels of heavy metals.[71] It was reported that heavy metals prohibit the biological functions of proteins by changing their native conformation via binding to them.[72] For instance, in the case of *Brassica juncea*, Cd-dependent modifications in beta carbonic anhydrase initiate the photorespiration improvement which might protect photosystem from oxidation.[73] The adjustments produced by Cd disrupt the alleviating communications associated with the modifications in the tertiary assembly thus causing the functional loss of that protein; fallout dysfunction of protein provokes the risk of protein aggregation.[74] The signals from heavy metals are recognized by receptors, and receptors transduce signals through cAMP, pH, etc. causing modifications in electron transport machinery

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of the cell, culminating in excess production of ROS that damages the macromolecules and thus creating oxidative stress (OS) within the cell. Moreover, the plant hormones occurrence, and indicators acquired by them start a cascade of signal transduction entraining a gibberellic acid-mediated GA-GID1-DELLA signaling pathway, haem oxygenase, and two transcription factors induced by brassinosteroids (BES1 and BZR1). Heavy metals stimulate extreme accumulation of ROS in plants. This process damages the cellular macromolecules namely proteins, culminating in physiological and metabolic disorders in cells or even cell demise. These parameters instigate manifestation of the nuclear genes encoding defense proteins, comprising heat shock proteins and metal transporter proteins and transcription factors. In fact, metal transporter proteins shield electron transport chains against heavy metals by controlling their absorption. Defense proteins defend plant against ROS in heavy metal stress. Toxic MIs (at cellular level) induce OS via generating ROS by promoting DNA impairment and/or weaken the DNA repair mechanisms, hamper the membrane functional reliability, nutrient homeostasis and disturb protein function.[75] The fate of ROS in the cellular system depends upon the result of many multifarious procedures which contribute to signaling cascades, anti-oxidative system, and redox alterations. It seems that OS happens, when the creation of ROS surpasses that of the scavenging capacity of antioxidants.

The main mechanism for the detoxification of heavy metals is the biological synthesis of metal fastening cysteine-rich peptides which function to immobilize, detoxify and sequester MIs. It was reported that in stress environments, MIs intensely influence cellular protein homeostasis by affecting their folding procedure and incite the aggregation of non-native or nascent proteins, culminating in the endoplasmic reticulum (ER) stress and diminished cell viability.[76,77] It was revealed that heat shock proteins acted as control mechanisms, which were specially articulated under stress to sustain healthy and functional proteomes. The impaired proteins that fail to attain

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their native conformations were degraded via the ubiquitin proteasome process, called as ER-associated degradation or via autophagy to curtail the buildup of misfolded proteins in cells.[78]

Plant cells have numerous adaptive mechanisms to manage additional MIs and employ detoxification mechanisms to prevent their involvements in undesirable toxic reactions. Firstly, plants prevent or control uptake by confining MIs to the apoplast via binding them to the cell wall or to cellular exudates, or by constraining lengthy transport.[79] Further, at higher concentrations, cells trigger a complex system of cleansing and storage approaches including chelation of MIs with metallothioneins (MTs) and phytochelatins in the cytosol, sequestration and trafficking into the vacuole via vacuolar transporters.[80] Phytochelatins (small cysteine-rich oligomers) produced at the initial stages of metal stress, has a vital role in facilitating plant tolerance to heavy MIs.[76, 81] Moreover, these oligomers have additional roles in plant cells including their participation in crucial MI homeostasis, sulfur metabolism and antioxidant mechanisms.[82] It was reported that over-expression of phytochelatin synthase (PCS) gene did not always bring about an increased tolerance to heavy metal stress in plants. It has been demonstrated that phytochelatins production was enhanced by 2.1-folds, when compared to wild-type plants.[83] Furthermore, additional phytochelatins levels in mutant plants increase the buildup of heavy metals without expanding plant tolerance.[84]

In addition to chelation, the stabilization and accumulation of heavy metals in the vacuole via production of high molecular weight complexes with phytochelatins were reported.[85] It was shown that the arrested MIs were transported from cytosol to the vacuole for appropriation via transporters. In plants, vacuolar confiscation is an important mechanism to heavy metal homeostasis, which is directly steered by ATP-reliant vacuolar pumps (V-ATPase and V-PPase) and a collection of tonoplast transporters.[86] De novo transcriptome and RNA-Seq examination

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demonstrated that various contender genes that encrypt heavy metal ATPases (HMAs), zinc iron permeases (ZIPs), ABC transporter, and natural resistance-associated macrophage proteins (NRAMPs) contribute to cellular metal transport and detoxification.[86]

Intracellular cysteine-rich major metal-binding proteins that occur naturally (MTs) were used by cells to impound, detoxify and immobilize, MIs.[87] It was revealed that by using MTs plants protect themselves from stress-induced oxidative damage. MTs participate in sustaining the homeostasis of vital transition MIs, appropriation of toxic and hazardous heavy metals, and defense against intracellular oxidative damage caused by stress.[88] Investigations have revealed that plant MTs have participated in MI homeostasis, especially for Cu, during both vegetative senescence and growth. Furthermore, it was demonstrated that MTs-deficient mutants accumulated 30% and 45% less Cu in root and shoot, respectively compared to the wild-type, while there were no clear disparities in the life cycle amid wild-type and quad-MT mutant plants under varied growth situations.[89] Transgenic plants overexpressing MTs genes demonstrated modified metal distribution or accumulation approaches, and were scored for enhanced metal tolerance.[90] It was reported that insufficient data existed about the precise mechanisms for transport of metals-MT complex to the vacuole from the cytoplasm.[91]

Plants characteristically respond to stress by eliciting the activation of the genes participating in cell death and/or survival in polluted environments.[92] In this activity, plant response universally involves a collection of genes, commonly named as stress genes, that were induced to produce a group of proteins termed heat shock proteins.[93] Under stress conditions, the induced synthesis of heat shock proteins plays a vital role in upholding the cellular homeostasis by supporting precise folding of stress accumulated misfolded and nascent proteins, by

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promoting discerning degradation of misfolded or denatured proteins or circumventing protein aggregation.[94 71]

By using proteomics, researchers can recognize the operative genes or proteins participating in the reactions of plants to heavy metal stress at molecular levels.[95] Transcript investigations of several plant species demonstrated that HSP70 was highly expressed under a varied metal stress levels.[96] HSP70 chaperones, alongside their co-chaperones such as DnaJ, render a group of leading cellular machines to avert accumulation of freshly produced proteins as aggregates and safeguard the proper folding of protein during their transfer to the terminus.[97, 98] It was reported that the induction of HSP70 limited the proteotoxic symptoms of MIs and helped the detoxification and sequestration of such ions by MTs.[99]

Cai et al.[100] demonstrated that heat shock proteins-caused metal tolerance in plants had a robust correlation with *N*-acetyl-5-methoxy tryptamine, (melatonin) generation, which, in turn, was controlled by heat-shock factor A1a (HsfA1a). Consequently, these findings proposed that the inducible heat shock proteins were critical and important for fitness in both, normal and in unpredictable environment. Moreover, in another research, Xu et al.[101] reported that heterologous expression of AtBiP2 protein in BY-2 acted as a damaging regulator of Cd-induced ER stress and programmed cell death. Further, Guan et al.[102] reported that ER chaperone binding protein operated as a positive regulator in Cd stress tolerance scenario. The transcript level of glutathione (GSH) gene was examined in LcBiP-overexpressed tobacco to examine the mechanism.[78] Liu et al.[103] reported a plant-specific component of ER-associated degradation system in Arabidopsis. It was demonstrated that EBS7 (methanesulfonate-mutagenized brassinosteroid insensitive 1 suppressor 7) interacted with the ER membrane-anchored ubiquitin ligase AtHrd1a, one of the main constituents of the Arabidopsis ER-associated degradation

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mechanism, whose mutation subverts AtHrd1a to relegate polyubiquitination. Further, Van Hoewyk [104] showed that Arabidopsis HRD1 and SEL1L mutant plants presented reduced tolerance to selenate (Se) stress. As-Se toxicity produced both OS and protein misfolding because of the replacement of a cysteine to Se-cysteine,[105] whereas selenium augments Cd tolerance in tomato plants.[106]

It was revealed that the manifestation of polyubiquitin genes under stress situations was one of the vital signs that the ubiquitin proteasome process participated in regulation of plant heavy metals stress tolerance.[107] The genome-wide transcription investigation of rice plants demonstrated that smaller concentrations of Cd conduct induced polyubiqutin expression in shoot and root.[108] Under extreme situations, over-expression of genes participated in ubiquitin proteasome process cascade, thus enhancing tolerance to manifold stresses without any undesirable effects on the developments and growth in plants.[109] The heterogeneous expression of rice E3 ligase enzyme synthesis RING domain OsHIR1 gene in Arabidopsis was reported to be reduced with buildup of As and Cd in both, shoot and root.[110] Furthermore, Lim *et al.*[111] demonstrated that ubiquitin ligase enzyme or the E3 has been a significant controller for the elimination of abnormal proteins under metal-induced stress. In another study, the expression profile Investigation of tobacco seedlings after exposure to five various heavy metals (Ni, Cu, Zn, Mn and Cd) treatments revealed that amongst the 30 ATGs genes, 18 ATGs genes were upregulated by two folds as a minimum one heavy metal. It was reported that among the 18 ATGs, 11 ATGs were usually up-regulated in seedlings by all five metals, and the manifestation was more responsive to zinc treatment than the rest.[112]

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4. Plant molecular farming (PMF): production of therapeutic proteins

Secondary metabolites have significant biological and ecological functions in plants, particularly advantageous is their role in chemical defense because of their antioxidative and antimicrobial activities.[113] Thus molecular farming is used for the large-scale production of valuable secondary metabolites. In addition, metabolic engineering tool can be used to overwhelm the bioactive-compounds availability limitations from medicinal plants and to improve productivity beneficial to both bioprocessing and molecular farming.[113]

The use of whole plants or in vitro cultured plant cells/tissues for the synthesis of desirable recombinant proteins (RPs) (pharmaceuticals and industrial proteins) is termed molecular farming, an economically feasible approach to production systems such as mammalian and microbes cells cultured in large-scale bioreactors.[4,114] Gene transfer technologies such as *Agrobacterium tumefaciens* mediated transformation or a particle bombardment (physical delivery) has been deployed to generate stably transformed plant or transient expression. Thus using plants for the preparation of recombinant non-pharmaceutical and pharmaceutical proteins, the formation of human serum albumin and antibodies were among the first examples of molecular farming.[115,116] Plant-based reactors have several advantages, most importantly: i) lower cost in maintenance, ii) competence to implement modifications to eukaryotic post-translational machinery function, iii) lower risks of contamination from animal pathogens, and iv) being amenable to large scale manufacturing process (Figure 1).[117]

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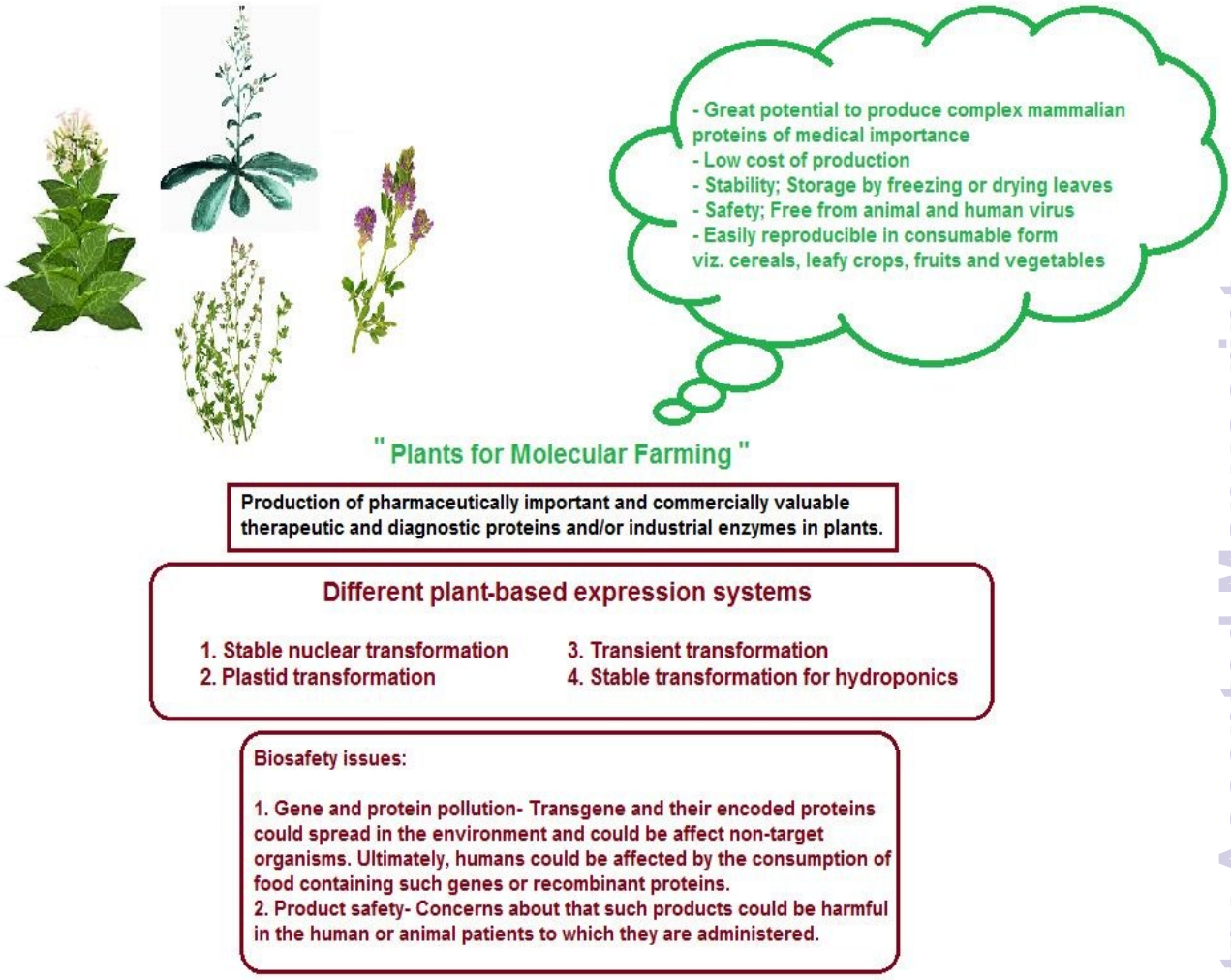

Figure 1. Plants for molecular farming: advantages and biosafety issues

The tissue-restrictive promoters can be applied for the production of RPs via selective expression of therapeutic genes in target organs of a plant. This kind of promoter is chosen due to its greater yield than constitutive promoters and accumulation in sink tissues with higher stability.[118] In addition, proteins might be specifically recruited to the cellular compartments such as the plastids, apoplast, cytosol or the endomembrane lumen by particular peptide tags. The ER, the first organelle in the secretory pathway, is transmitted by plasma membrane, vacuolar, lysosomal and secretory proteins to their port of depot placement, and exports of proteins to the

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Golgi apparatus for sorting to their final destination after ensuring the correct folding and assembly of polypeptides.[119] Secretory proteins, that mainly synthesized by ER-bound ribosomes, are directed to the ER by including an *N*-terminal signal peptide in the growing polypeptide chain. Plant seed is an ideal platform for production of desired biopharmaceutical proteins like bioactive peptides, vaccines, cytokines, antibodies, and so forth.[120] Its advantages include the high yield and high stability of RPs accumulated in seeds which are stable for years at ambient temperature without deterioration in bioactivity. Several types of plant seed-specific promoters have been employed to target expression in particular tissues in seeds including whole seed expression promoter, aleuronic layer-specific promoter, endosperm specific promoter (predominantly expression in whole endosperm subaleurone layer, inner starchy endosperm zone) and, transfer cell specific-, and embryo expression-promoter. Plant cell and its organelles, seed anatomy and protein targeting organs can be fully exploited to accumulate high yield of a recombinant protein in an appropriate compartment.

The roots, stems, leaves, seeds or *in vitro* cultures of cells and tissues obtained from one of these organs can be used to selectively express the RPs. In the vegetative organs (root, leaf and stem) cytosol, apoplast, vacuole, and chloroplast can be used to target the RPs which can also be maintained in the ER. Targeting plastids result in high recombinant protein yield but lacks some of the posttranslational modifications like glycosylation. The RPs can be directed to either the embryo or the endosperm in the seeds.

The following plants are desired targets for the production of various therapeutic proteins: *Nicotiana tabacum* and *N. benthamiana* (tobacco; model plant), *Daucus carota* (carrot), *Medicago sativa* (alfalfa), *Lactuca sativa* (lettuce), *Musa paradisiaca* (banana), *Zea mays* (maize), *Glycine max* (soybean), *Solanum tuberosum* (potato), *Solanum lycopersicum* (tomato),

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Oryza sativa (rice), and *Triticum aestivum* (wheat).[121] Plant can be genetically modified in order to produce chemicals and pharmaceutics for biomaterial purposes. The advent of plant molecular farming (PMF) had re-invigorated and elicited global interests in the production of precious natural bio-active molecules, pharmaceutical proteins and more recently nanostructures. Several authors have emphasized the application of plants, algae and yeast as reliable sources of carotenoids, chlorophyll, long-chain polyunsaturated fatty acids, phycobiliproteins, collagen, and enzymes.[122-124]

Plant polymers with access to only twenty amino acids do not have the diversity of the polymers that are synthesized using present chemical polymerization techniques. Nevertheless, using the gene transfer technology, it is possible to determine the molecular weight, amino acid sequence of the protein and generally have a control over the physicochemical properties and functionality of the protein, which is hard to achieve using the chemical polymerization techniques. One of the main limiting factors of plant-based biomaterials is the sufficient and economical production of the material in order to design its functionality and possible applications.[125] However, significant advancements in the field of biotechnology and gene transfer, has helped circumvent the limiting factors. Transgenic plants products are low-priced and safe with easy production processes compared to the products of the expression systems in animal or microbes. Despite the creation of different valuable pharmaceutical antibodies and proteins in transgenic plants, there are issues regarding public acceptability and possible risk of gene escape.[126] It is expected that in the near future plant-based biomaterials will experience tremendous advancements as PMF depends on genetic manipulation of plants by transferring gene via viral vectors or introduction to nuclei and chloroplast genomes.[127] This technique can be

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an efficient alternative to biomaterials such as collagen and gelatin that are normally extracted from animal tissues (such as bovine hide).

4.1. Production of collagen

Fibrous proteins with long repeated amino acids such as collagen and elastin have some significant physical properties including elasticity, toughness, and strength that distinguish them from other short block proteins making them interesting for biomedical applications.[128] Collagen, for example, is a repeated sequence of GlY-X-Y where X is proline, and Y is hydroxyproline, elastin is also a repeat of Val-Gly-Val-Pro-Gly.[125] The cost of these biomaterials have soared apparently due to increase in demand and risks associated with animal derived biomaterials such as contamination with pathogens and enhanced disease transmission risks.[129] Other factors that make plant-derived substitutes to be in high demand include safety, less prone to risk, relatively low extraction cost, easy storage of products and ease of scale-up .[127, 129,130] However, low yield of products, inconsistent final proteins quality and the presence of impurities in the product are some of the major drawbacks of PMF[131] which has hindered the approval issues and therefore a wider distribution and usage in pharmaceutical and food industry.[131,132]

Collagen represents 30-40% of the protein in the body which comprise 90% type I collagen and non-collagenous proteins like glycosaminoglycans. It accounts for 90-% of inorganic phase of the bone matrix.[133] A collagen molecule has three compartments of α chains which form a three-helix structure, this alpha chain consisted of repeating sequences of Gly-X-Y where X is proline and Y is hydroxyproline residue.[134] A correct formation of collagen triple helix depends on the presence of all these sequences. The network consists of well-organized parallel fiber or bundle. The collagen fiber is composed of long tough molecules known as

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tropocollagen comprising various peptide chains. Based on the amino acid sequence of tropocollagen different collagen can be produced. So far, thirteen assorted collagen types have been recognized. Type I collagen is the most plentiful form in the body which normally consist of two chains of α1(I) and one chain of α2 (I) of a homotrimer (α1[I])3.[135] The distribution and number of collagen fibril (approximately 100 nm in diameter) in tissue influence its mineralization.[136] It provides high-density filaments and forms layers in the bone. Collagen has been an important material for biomedical, cosmetic and tissue engineering products due to its unique role in tissue repair and restructures. It has a prime role in tissue engineering and is an integral part of extracellular matrix (ECM) tissue engineering with proven advantages for repair and restoration of the injured tissue[134, 137], and has been used for skin application, bone, and ocular regeneration and has widespread applications in tissue engineering.[138] It is normally processed into sponges for tissue regeneration application but also is applied in hydrogel, electrospun fiber, film, sheet, disks, pellet, NPs and tablet forms.[139] Ever since 1981, bovine-derived collagen has been in use in biomedical industry, it has been a standard to which all other soft tissue reinforcement materials are related to. However, over 35 years after, it is nonetheless not the ideal material because it is costly and can induce allergic reaction in tiny percentage of the patient.[140] Collagen is commercially extracted from animal hides (porcine skin, bovine tendon) or scare cadaver [141] which has been reported to cause an unwanted human response in up to 10% of the treated patients.[142] Allergic reactions such as swelling, redness and itching have been reported for the area of collagen replacement.[142] In these rare cases, patients have to treat with anti-allergic drugs such as cyclosporine.[140]

Despite these reactions which are normally resolved in due course but other serious drawbacks such as non-incomplete adsorption and implant functional failure have also been

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observed for animal-based collagen implants.[140] A skin test is normally carried out as a general protocol to prevent the occurrence of hypersensitivity to collagen, however, the process normally takes up to four weeks, which limit the collagen application in an emergency situation.[143] Apart from the hypersensitivity reactions and time consuming process of skin test, transmission risk of prion to human cell is another issue in application of animal derived collagen. Therefore, some standards such as ASTM F 2212 - 08 [144] limited collagen extraction only to some closed herds or from animals raised in a country that have no occurrence of bovine spongiform encephalopathy (BSE) like New Zealand.[145] Aside from bovine-derived collagen, human-derived collagen also has some drawbacks. The age, genotype, ethnicity and environmental condition of the donor effect on biophysical properties of the derived collagen and result in a big variability in the final quality of the product on the market. It has been reported that with increasing age, collagen gets deprived of it's swelling, elasticity and acid solubility properties due to the intermolecular cross-linking.[146] Cultural reasons and the variation between batches also add to the concern and so accelerating requirement for the collagen-based biomaterials, have paved way for the development of alternative sources for the production of collagen.[141,147] With this development, recombinant collagen has been produced using yeasts, mammalian cells and bacterial systems.[148] Pro-collagen (commercial collagen) is prepared from domesticated animals, including pigs and cows and has an increasing global demand.[149,150] Inauspiciously, it is liable to harbor human pathogens, comprising prions or viruses.[149] As a viable alternative, tobacco plants have been used to efficiently express human recombinant type I pro-collagen using transgenic technology.[141] Figure 2 shows a schematic overview of production of recombinant collagen using transgenic tobacco leaves. Nonetheless, hydroxylated collagen can be produced in relatively large quantity using transgenic plants.[147] In early studies, Ghosh et al.[151] reported a

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detectable amount of collagen in plant nuclei[151] although no purification or extraction of the collagen was performed. Plant produced collagen was found to be pure, free of contaminants and with a high content of hydroxylated proline and lysine similar to the human-derived collagen; vacuoles were targeted for expression of the procollagen that consequently resulted in the formation of thermally stable, and dense packed mature collagen with triple helix structure.[141] The alpha helical structure was preserved and the extracted collagen showed characteristic properties of the tissue-derived collagen such as high surface area, binding sites and high water holding capacity. The plant-derived collagen showed comparable properties in terms of processability and pharmaceutical application and efficiency.[148]

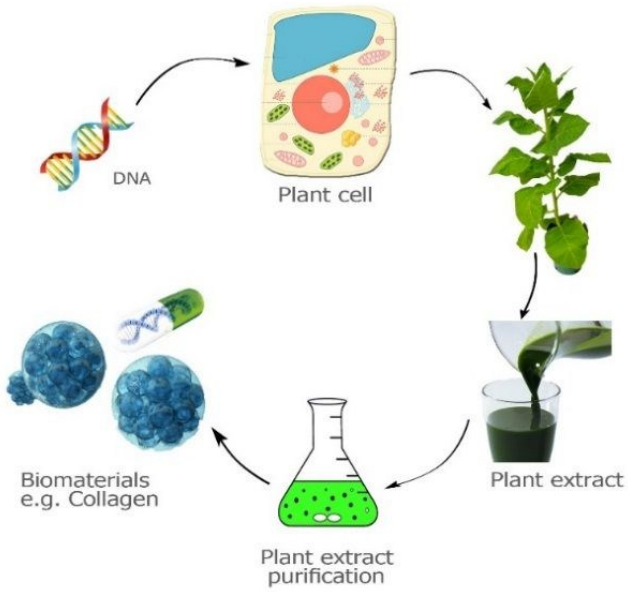

Figure 2. Schematic overview of production of recombinant collagen using transgenic tobacco leaves.

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Willard *et al.*[130] compared the plant-derived human collagen (PDHC) and bovine derived collagen (BDC) using electrospun and freeze drying process and found that PDHC was processed and ready within 20 min for electrospinning which was much faster than 48 h required for bovine collagen, additionally, electrospun fibers of the PDHC were thinner and more round and uniform. Further, the PDHC showed superior solubility in acetic acid and better biocompatibility and cell proliferation at a concentration >12% (W/V) when compared to bovine samples. However, bovine collagen constructs showed better mechanical properties probably due to higher viscosity and higher collagen content of the bovine sample at the same concentration (80% vs 97%), and, therefore, scaffold with thicker fiber size could be produced.[130] Nevertheless, the mechanical characteristics are more related to good cell growth, proliferation, and epidermal formation than the basic properties of the scaffold.[152,153] In general, the mechanical properties (ultimate tensile strength) of the electrospun collagen fiber (0.01-0.7) [154] is much lower than 2.7-10 Mpa[155] for human skin. Therefore, a technique such as using high strength polymer in production of biocomposite[154] is currently researched to enhance the mechanical characteristics of the fiber. The wound healing properties of the PDHC (Vergenix FG) was assessed by Shilo et al.[156], using pig and rat as big and small animal models and found a faster healing process in the animals treated with PDHC. It has been reported that wound shrunk after 24 hours, and a 66% closure recorded after 6 days in the rat model while in porcine 95% wound closure was observed after 21 days while control groups showed a closure of 68%.[156] Stein et al.[147] targeted vacuole proteins for expression of collagen and showed that up to 200 mg of recombinant heterotrimeric collagen type I (rhCOL1) can be extracted per 1 kg of fresh tobacco leaves. Authors concluded that the yield was significant compared to 10-100 mg[157] that was obtained via targeting the nucleus in plants. Tobacco plants have been used for many years

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for production of plant-derived proteins, because this plant can be easily regenerated in tissue culture within 6-8 weeks and readily amenable to transformation. Tobacco now gains a well-established plant host status for the expression of proteins.[3,158] It has high yields of biomass, rapidly scalable and also is not considered as a food or feed crop and therefore, has a lower risk of transmission of transgenic proteins that can contaminate food or feed chain. However, tobacco contains a high content of alkaloids especially nicotine, which needs to be removed during processing. Therefore, some other crops such as lettuce and alfalfa have been suggested for their possible application in molecular farming.[3]

There are 25 different types of collagen in human base on DNA and/or protein sequence information.[159] Ruggiero et al.[160] transformed tobacco plants using cDNA to encode human preproα1 chain for collagen I, and suggested that triple helix of collagen can be produced, despite that the final product was completely transformed to collagen but the product has lower thermal stability due to lack of prolyl-hydroxylation in the plant. Hydroxylation of proline residue in the sequence of Xaa-Pro-Gly of the helical structure of collagen is required for proper functionality of this ECM I physiological environment.[161] In order to overcome this drawback, in one study by Merle et al. [162] hydroxylated homotrimeric collagen was produced in a tobacco plant using human type I collagen and a chimeric-4-hydroxylase (P4H). This modification and co-expression with animal-derived enzyme were thermally stable up to 37°C. In addition to thermal stability, hydroxylation is essential for binding of collagen to integrin α1β1 and platelet receptor glycoprotein V1 for activation and aggregation of platelets.[163] In another study, maize seed was applied for the preparation of human collagen type 1 α-1 with the higher percentage of (Hyp) (18%) via co-expression with human P4H; co-expression of rP4H increases the thermal

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stability of rCIa1 with the reported yield up to 12 mg/kg.[161] Quantity and hydroxylation of some plant-derived human collagen I are summarized in Table 1.

Table 1. Plant names, quantity and hydroxylation of some plant-derived human collagen I

Plant name	Quantity/tissue	Hydroxylation (%)	Ref
Tobacco	30mg/kg dried leaf	0.53	[160]
Tobacco	N/A	N/A	[164]
Tobacco	0.5-1 mg/kg leaf		[162]
Tobacco	200 mg/kg fresh leaf	7.55	[165]
Barley P1 cell	2-9 µg/cell culture	NA	[166]
Barley seed	Below detection	2.8	[167]
Maize seed	200 mg/kg seed	2.01	[168]
Maize seed	3 mg/kg	1.23	[169]
Maize seed	15.9 mg/kg germ (CIα1) 49.6	NA	[169]
Maize seed	12 mg/kg seed (CIα1) 4 mg/kg seed (CIα1-OH)	18.11	[161]
Tobacco plants	2 percent of the extracted total soluble proteins	NA	[130]
Tobacco plants	1 g/kg dry tobacco leaves	7-10%	[141]

4.2. Production of gelatin

Gelatin is a hydrolyzed form of collagen with wide applications in food, beverage and pharmaceuticals where it is largely used for capsule production. Over the years, the demand for gelatin has been on the increase to which the conventional method of production, based on microbial bioreactors, cannot sustain. Consequently, there is a demand for a novel and proficient means of gelatin production. Transgenic plants can be suitable and cost-effective alternatives for the production of gelatin; reportedly, transgenic plants have a limitless production capacity and relatively low regulatory capital.[159] Gelatin-like collagen is an extracellular matrix component

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that has an important role in attachment, growth and proliferation of the cells, by providing a proper substrate for different cells to attach and grow. It has been reported that recombinant gelatin that expressed in Pichia (*Pichia pastoris*) can provide an ideal support for Vero cells similar to the gelatin samples acquired from commercial bovine.[124,159,170] Montagnon et al.[170] used a 50 KDa gel fragment of recombinant gelatin to coat beads and observed that the tested beads were populated by vero cells suggesting that recombinant gelatin can replace bovine gelatin. However, the low productivity is a major constraint to use plant cell cultures for the preparation of RPs. In a related study, barley (*Hordeum vulgare*) was investigated for the production of gelatin as a possible alternative to tobacco plant[166] as it can grow over a broad environmental range, store large quantity (15%) of proteins with no risk of gene drift because barley is a self-fertilizing species. However, the intracellular accumulation of Collagen I alpha 1 (Cla1) was only 2-9 µg/l requiring further improvement of the process technique for supplying the material.[166]

4.3. Production of elastin

Elastin, a biodegradable and biocompatible non-toxic protein with strong elastic properties, is present in connective tissues such as ligaments and plays a critical role in normal routine stretching and contacting of the tissue. This protein enables skin to return to its original position after the pressure has released. Elastin is repeats of the Val-Gly-Val-Pro-Gly and synthetic protein made from multiple repeats also showed elastic properties.[125] Polypentapeptide of elastin also has been used to prevent postoperative adhesion in a peritoneal wound model.[125,171] Other biomaterial applications of this protein-based biopolymer are artificial pericardia, wound bandage, intelligent drug delivery and absorbents.[125,172] Expression in *E. coli*

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of a manmade protein comprising 121 repeats of the Gly-Val-Gly-Val-Pro peptide was demonstrated to culminate in buildup of the polymer in inclusion bodies.[173,174] However, only low polymer accumulation was obtained when the same expression performed in the fungi *Aspergillus nidulans*,[175] the reasons for these lower accumulations are not clear but chloroplast is the predominant location within cell for formation of this protein. Guda and Daniell [172,174] expressed 121 repeats of the elastin sequence in *E.coli* and the polymer accumulation was observed in inclusion bodies. In various studies, Guda et al.[172] and Zhang et al.[176] expressed elastic biopolymer in tobacco with amino acid sequence of GVGVP and showed that using chloroplast for the expression is 100 fold more efficient compare to nuclei for the production of elastic biopolymer.[172] Hemoglobin-based blood substitute has also been developed using tobacco plant, Dieryck et al.[177] co-expressed α and β globins of the human hemoglobin HbA in transgenic tobacco plants and produced tetrameric hemoglobin and the authors believe that other species as well as tobacco can be applied for the same purpose with simpler modification and higher yield for production of hemoglobin.[177] Despite the promising outcomes of the recombinant plant-derived collagen, however, this technique needs further optimization to overcome the obstacles, including the low yield, high cost, and lack of some critical enzymes in the system for synthesizing the collagen with comparable properties to the animal-derived collagen. Therefore, until these issues addressed, its use over the animal-derived pathway will be limited. However, there are some niche markets such as collagen type 2 whose production from animal is difficult in larger quantities; there are some active companies such as Collplant (www.collplant.com) that have some plant-derived collagen product in the market but the products do not have approval for sale in the European or the US market. This niche field might

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be applied as the experimental base that might help to develop the technology and get the plant-derived strategies close to large scale production and possible clinical application.[148]

4.4. Production of recombinant anti-cancer monoclonal antibodies

Even though artificially production of antibodies by means of mammalian cell culture systems have acquired a great success in neutralizing a wide-ranging of diseases, high level of production costs together with long period of manufacturing time have challenged their scalability and utility.[178] Plants do not produce antibodies against their viral, microbial and fungal enemies. Naturally, antibodies are produced by human's and other mammal's immune system. However, thanks to genetic engineering techniques and by introducing the corresponding coding sequences, plants are now able to artificially produce antibodies in a safe, low cost and convenient manner. It is also to be noted that in comparison to other expression platforms such as bacteria and yeasts, plants-produced glycoproteins are remarkably similar to those produced by mammalian cells from the *N*-glycan composition viewpoint.[179] There are five types of immunoglobulins in mammals, of which some are structurally different and additionally complex than typical mammalian serum-type immunoglobulin G (IgG).[180] For instance, IgG could be expressed only by introducing two foreign genes into the either plant chloroplast or nucleus genomes; whereas four genes should be transferred for producing IgA since it assembles as IgG-like tetramers. Because of being foreign proteins, initially, there was a serious concern on antibody production in plants addressing whether IgG molecules could be functionally expressed and assembled to multi-structure complexes. For scrutinizing this, tobacco plants were separately transformed with genes coding heavy and light chains of typical IgG and monitored for polypeptide expression. Furthermore, the two separate lines were

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crossed and their progenies were tested for assembling the monomeric structures and forming the tetrameric structure. As a result, tobacco plants successfully produced catalytic IgG in a correct monomeric and tetrameric structure.[115] The potential of plants for affordable production of biopharmaceuticals has been recently re-certified by producing a group of valuable materials including ZMapp™ antibody cocktail against Ebola and the chloroplast-produced virus-free oral booster polio vaccine.[181,182]

The numerous types of antigens are specifically recognized by antibodies whilst the structure of antibodies is mostly conserved. By identifying the molecular mechanisms behind the diversity of natural antibodies and by isolating the antigen binding variable regions through polymerase chain reaction (PCR) or other synthetic procedures and embedding them on a predesigned framework, researchers are able to design and produce synthetic recombinant antibodies for a variety of aims in medicine, agriculture, and industry. This ability makes antibodies strongly useful to be employed in the systems biology procedures such as diagnostic, reagents and therapeutics.[183-185] It appears that hijacking PMF as a rapid and scalable technology along with these kinds of advanced techniques will, therefore, help humans to overrule the future challenges. Three kinds of applications for plant-derived antibodies have been achieved. Extraction and purification of plant-produced antibodies for healthful applications was the initial conception in PMF. During this approach, plants are simply engineered to produce the Antibodies of Interest (AOI) and as mentioned earlier, because of addressing three kinds of obstacles ahead for preparation of RPs in traditional expression systems based on yeast and bacteria i.e., cost, scalability, and safety, plant-based platforms seems to be most promising.[186] As the second application, it has been demonstrated that plants can be engineered to produce predesigned antibodies for agricultural applications like those to

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employ as fungicides and bactericides. In this manner, synthetic plants are hijacked to produce AOI for act as plant artificial immune system against enemies like pests, bacteria, and viruses. The purpose of this technique is to produce the synthetic Eco-friendly pathogen-resistance plants so as to reduce the use of chemical bactericides and fungicides.[187] The scFv antibody was successfully expressed in *Nicotiana benthamiana* and effectively neutralized the *Artichoke mottled crinkle* virus so that the transgenic plants showed lower amounts of infection.[188] In another interesting report, a nano-body that confers strong resistance against *Grapevine fanleaf virus* (GFLV) was identified whose stable expression in grapevine and *Nicotiana benthamiana* showed resistance against a broad spectrum of GFLV isolates.[187, 189] As the third approach, synthetic antibodies which are produced intracellularly in plants can be employed for metabolite engineering or pathway regulation since the binding of the synthetic antibodies to a specific intracellular molecule has the potential to block, suppress, alter, destroy specific cell type or even enhance the activity of the specific targets. The production of intracellular antibodies with the aim of altering natural biological processes is also known as intrabodies; their use for targeting cell specific molecules as an effective, and more specific alternative to genome-based knockout procedures such as RNA silencing and virus induced gene silencing (VIGS) is commencing to be resuscitated by overcoming some technical obstacles affecting antibody production in plants.[190]

Various kind of materials such as synthetic particles (*e.g.,* polymers, gold and lipids), and biological material (*e.g.,* viruses, nucleic acid sequences, and polypeptides) have been employed for nanoparticle-based vaccines. Of these, because of having high affinity and efficiency to find their targets, antibodies have been employed as scaffolds for NPs.[191] In addition to polypeptide based materials which are the typical targets of antibodies, they target

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various range of materials like viruses, carbohydrates, and nucleic acids.[192] Antibodies are easy to engineer and they both combine safety and the ability to elicit a strong immune response because of their designable size and shape. It appears that the production of predesigned antibodies in plants is the complement in producing bio-nanomaterials.[193] Recently, plant-based production of the core protein of the hepatitis B virus (HBcAg) fused to 'tandibody', a camelid nano-body, has been developed. HBcAg naturally assembles in virus-like particles when expressed in plants and it has a major insertion site allowing for the integration of foreign sequences which finally emerge in tip of the particle. The antigen binding activity of the nano-body allows the particles specifically to find their targets and provide a platform for producing highly effective immunogenic vaccines. The approach is completely flexible since the specific part of it could be easily re-designed for various targets.[194]

Kim et al.[195] reported patterns for the glycosylation and expression of monoclonal antibody CO17-1A (as colorectal anticancer agent) identifying the tumor-associated antigen GA733-2, expressed in human colorectal carcinoma cells in the stem and leaf tissues of primary (0 cycle), secondary (1 cycle), and tertiary (2 cycle) growths of seedlings achieved from the stem cut of T2 plants.[195] Consequently, in the 1 and 2 cycle growths, the stages for floral organ creation (35 days) was briefer than that (100 days) for the 0 cycle growth. The genes of light and heavy chains of monoclonal antibody CO17-1A were at the top, middle, and basal portions of the stem and leaves realized from the 0, 1, and 2 cycle plants. The protein amounts in the stem and leaves tissues from the 1 and 2 cycles were comparable to those in the tissues from the 0 cycle. The glycosylation level and pattern in the leaf and stem did not modify dramatically over the different cycles. Analysis showed that obtained mAbs CO17-1A from

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stem and leaf tissues of the 0, 1, and 2 cycles displayed analogous binding affinity for the GA733-2 antigen.[195]

Some important studies have focused on the optimization of recombinant protein expression levels, including monoclonal antibodies in transgenic plants, such as the application of plant codon-optimized genes, different promoter genes, silencing suppressors and assorted targeting approaches for recombinant protein accumulation.[196-198] Importantly, in one study, in order to find the best extraction method for recombinant monoclonal antibodies from tobacco plants, various factors have been evaluated including pH, temperature, mechanical disruption method, buffer composition, etc.[196] In this study, three various lines of transgenic plant were investigated to evaluate the parameters affected the ideal extraction of monoclonal antibodies accumulated at the plasma membrane, in the apoplasm or inside the endoplasmic reticulum. These parameters exactly showed critical influences on the initial selection of the expression approaches, and therefore should be checked, primarily. The application of small-scale methods which are adaptable for large-scale purification was a mainly critical consideration.[196] It has been reported that the optimal extraction method might be changed with IgG target location in plant cells, the antibody yield dependence on the employed physical extraction approach, and pH of the extraction buffer and temperature in each sample. The yield of production might be improved by adding detergent to the extraction buffer, but this process was reported to be dependent on the site of IgG accumulation within plant cells. For evaluation of the temperature effects, it was shown that recombinant proteins were susceptible to degradation by proteases released during the extraction procedure from fresh plant tissue. Therefore, extraction processes were normally conducted on ice or in liquid nitrogen. In this study, influence of the extraction buffer pH was evaluated in transgenic plant examines. Three leaf discs were ground in buffer of

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the same ionic strength with the pH varying from 2.8 to 10. Consequently, little or no functional IgG could be identified at pH 3-4. It was reported that pH about 5 to 7 was appropriate. When the extraction buffer approached the pI value of IgG1 (8-9.5), less monoclonal antibodies was obtainable. When the tobacco total soluble protein levels were measured, the amount of total soluble protein released at pH 7.4 was extremely higher than that at pH 6. Additionally, the amount of total soluble protein released at pH 7.4 was extremely lower than that at pH 8. Findings showed that IgG was optimally obtained at pH 5-6 for maximum IgG yield and minimal total soluble protein contamination.[196]

Despite the huge importance of plant-based systems in the production of antibodies, the major drawback in production of antibodies lies within a combination of both low level of expression and high level of proteolytic degradation which results as loss of end-product[199] (Figure 3). Low level of expression is not a specific matter for antibody production in plants because it is found in any other recombinant proteins either. There has been large number of attempts to efficiently increase the expression level; use of chloroplast genome instead of nuclear genome has been shown to incredibly boost the expression level.[200] In other attempts, the crucial role of expression cassettes elements on antibody expression level like codon optimization,[201] viral suppressors of RNA silencing (VSRs),[202] and the innovative enhancing insulators have been ascertained.[203]

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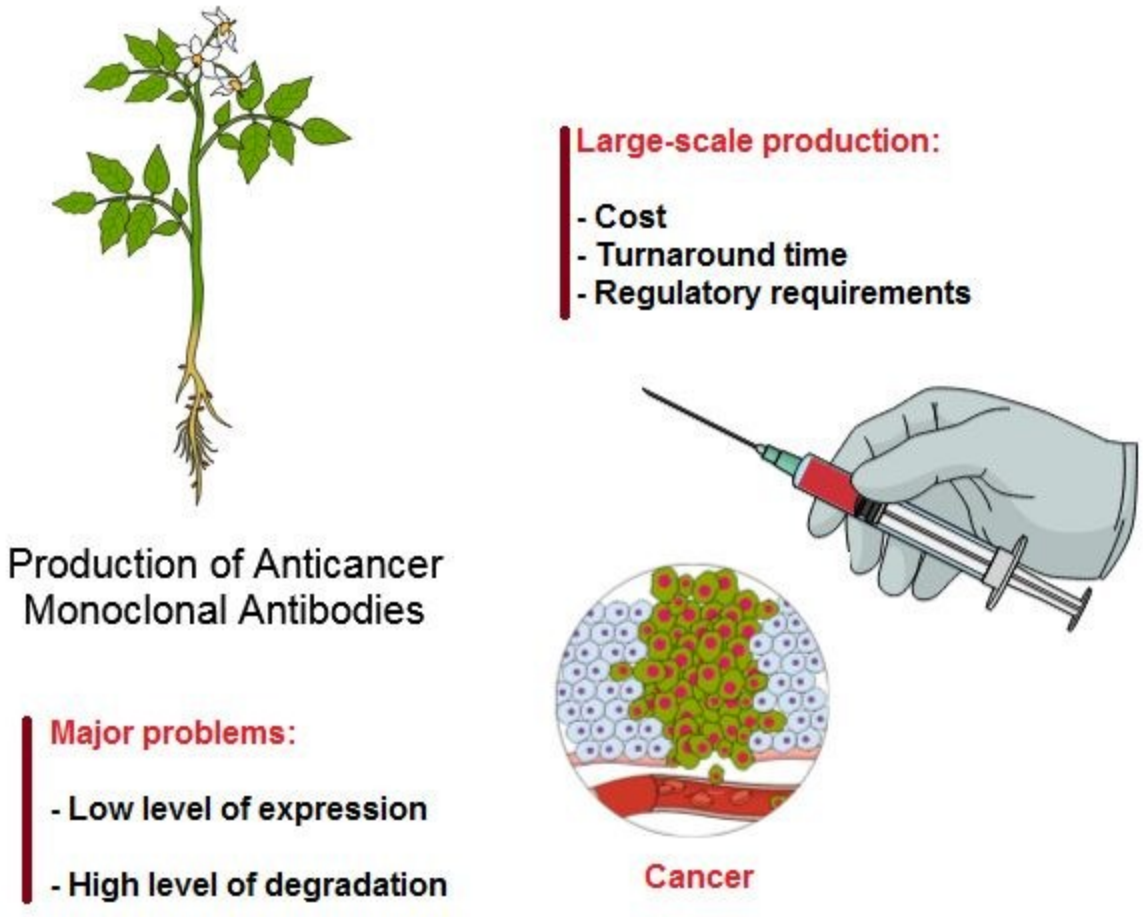

Figure 3. PMF and production of recombinant anticancer monoclonal antibodies

The production of truncated antibodies with additional and/or smaller than expected fragments has been reported in different tissues such as leaf, seed, and callus, and tuber of various plant species.[204] This truncation could be because of both mis-assembling which is the final step in antibody production procedure and the consequence of extracellular proteases activity during purification. Although it appears that the two mentioned phenomena happen at the same time, the major problem in alleviating the final product of the antibodies is the degradation through extracellular proteolytic activity.[204] Because the level of degradation is depended on host-antibody interaction, it is not easy, even though a lot of attempts have been

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made to develop a general role for preventing proteolytic degradation. Sharp and Doran [204] revealed the degradation patterns of a mouse IgG1 antibody in *N. tobacum* plants. They suggested that the degradation was most likely occurred during purification possesses in extracellular medium such as apoplast or during secretion from ER to Golgi. To reduce the proteolytic activity, several strategies have been taken into account such as confining the proteins of interest into the subcellular compartments like ER. The ER has been long accepted as a safe place for heterologous proteins accumulation which can easily derive by adding KDEL signal peptide that drives the proteins of interests into the ER.[205] Furthermore, by co-expressing the protease inhibitors with antibody of interest and targeting them into the apoplast, scientist have managed to boost antibody production.[206]

4.5. Production of recombinant anti-cancer vaccines

Vaccination against cancer diseases is a novel approach to generate tumor-associated antigens (TAAs) in plants. Various anticancer vaccines expressed in plants have been investigated and even clinically accepted for assessments. These vaccines can be produced in large scale. Tissue positions, plant growth conditions (such as, temperature, drought stress multidimensional stress, salinity, and soil nutrition) and harvest times influence the glycosylation structures and protein expression levels of cancer vaccine proteins in plants. Selection of the best plant species for steady alteration and management of environmental parameters which influence plant fitness conditions are very critical.[114, 195, 207-209] In one study, Lim et al.[210] demonstrated that colorectal cancer vaccine protein levels in stems and leaves reaped after flower fertilization were smaller than the plant material gathered before the blossoming period. As a result, the highest

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manifestation level of a colorectal cancer vaccine protein was reported in the 12 weeks after the *in vitro* plant seedlings were transplanted.[210]

Generally, several specific parameters might be considered in cancer vaccines production in plant expression systems. Suitable vaccine candidates might be chosen and aimed to produce potent immune reactions against disorders in order to efficaciously prevent and remedy diseases. Furthermore, it is vital to select suitable antigenic proteins which the immune system might target. Responses of immune system to cancer vaccines comprise systemic and mucosal challenge to a vaccine after its direct treatment via parenteral injection or mucosal surfaces, respectively.[211] Actually, administration of therapeutic cancer vaccines to cancer patients can induce the defensive capability of the immune system to unambiguously identify, assault, and destroy tumor cells. Moreover, these vaccines are dispensed to healthy populace to avoid cancer from occurring. Some important advantages of anticancer vaccines production using plant systems as well as related critical issues, are highlighted (Figure 4).

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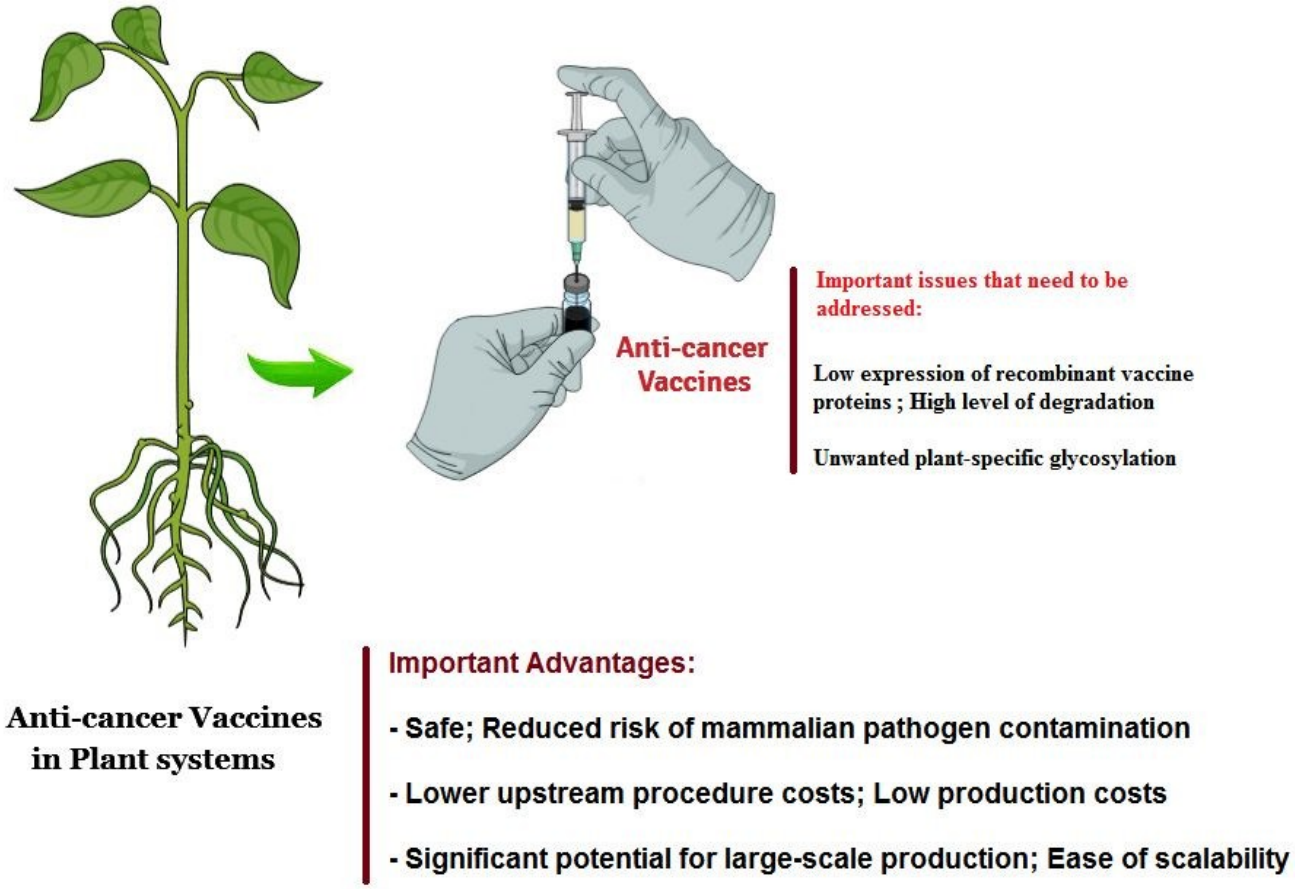

Figure 4. Anticancer vaccines production using plant systems: advantages and some important issues

Anti-cancer vaccines generated in plants might be positioned to appropriate antigenic proteins which the immune system can target. Tumor specific antigens (TSAs) and tumor-associated antigens (TAAs) are classified as tumor antigenic proteins. TSAs are precisely expressed on the tumor cells and trigger better immune responses than in the case of TAAs. It is challenging to recognize TSAs as vaccine candidates, and they are extremely scarce. TAAs are expressed on both normal and tumor cells and can induce a weaker immune response than in the case of TAAs, and they are frequently identified on tumor cells. Various anti-cancer vaccines were articulated in plants, and accordingly, non-Hodgkin's lymphoma, colorectal cancer, and cervical cancer were targeted.[114, 195, 207-209]

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One of the important candidates for anticancer vaccine production is E7 oncoprotein from Human Papilloma Virus (HPV). Massa *et al.* reported HPV16 E7 coding sequence (wild type or mutagenized sequence, E7GGG) as fusions to β-1,3-1,4-glucanase (LicKM) of *Clostridium thermocellum* and produced in *Nicotiana benthamiana* plants using a transient expression system. Consequently, both fusion proteins induced E7-specific IgG and cytotoxic T-cell responses and protected mice challenge with E7-expressing tumor cells, and thus could find use for prevention of tumor development.[209]

5. Challenges and opportunities

The main aim of the molecular farming of the biomaterials is to produce a large amount of secure and functional materials in an efficient way with low production cost.[127] Therefore, in addition to legal requirements, the success of using the plant-based biomaterial depends on its sufficient extraction at the required level in a sustainable yet efficient and economically way. Despite the number of different studies that explored various means of extracting protein polymer from plants, but there is still a gap regarding the validation of the current technologies in the context of scaling up the production of these plant-based biomaterials.[125] It was shown that the amount secured should be higher than 1% of soluble protein to make it commercially interesting.[212] In contrast to animal and microbial expression systems, transgenic plants have several advantages in terms of safety, cost and ease of production for fabricating therapeutic biomolecules. However, there are quite a few challenges, such as public acceptance, transgene escape, biosecurity, and public reception among others, but it is anticipated that in not too distant future, molecular farming will see substantial accomplishments with the precise and technical inquiries; significant concerns are summarized in Figure 5. Basic and original investigations are

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required for the commercial success of ensuing products in spite of some advances that have occurred in production of medicines in plants. The present problems include the low yield of proteins, the possibility of harmful effects on the function/performance of proteins due to the variances in glycosylation configurations, and the likely influence on the environment.[114, 208] The potential of plants to be exploited for the very-large-scale production of biopharmaceutical proteins (such as monoclonal antibodies) have been discussed by Buyel et al.[213] They reported on the potential market sizes and their corresponding production capacities, and available process technologies and scale-down models and how these can be applied to develop large-scale processes.[213] Furthermore, they reviewed comprehensively the extraction and downstream processing of plant-derived recombinant proteins.[214]

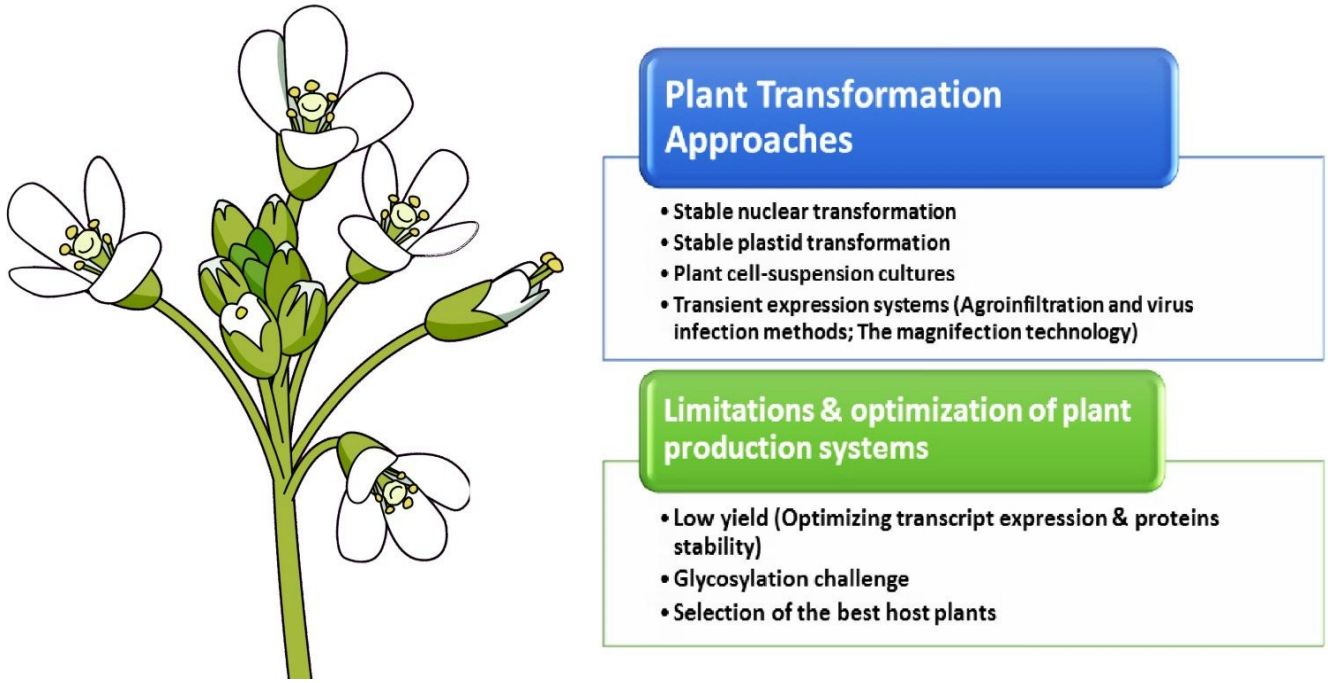

Figure 5. PMF and related significant concerns

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In production of recombinant human collagens, it appears that some important challenging fields need to be addressed such as insufficient post-translational modifications and low yields. Currently, in addition to conventional cancer treatments, active immunotherapy is being emphasized. Optimal combinations of antigens, adjuvants and delivery vehicles might be regulated and valuable approaches for overcoming tumor-associated immunosuppression should be improved. It appears that more elaborative investigations might be required in order to determine new predictive biomarkers and their prospective validation in the real-life clinical setting.[1, 25, 45, 207, 208, 215]

6. Conclusion

PMF is the generation of therapeutically significant and commercially viable secondary metabolites and RPs in plants, and its success is dependent on a genetic transformation of plants attained by the strategies such as viral vectors, methods of stable gene transfer, namely gene transfer to nuclei and chloroplasts. Nowadays, with scientific advancements in greener nanoscience, phyto-nanotechnology and biotechnology, gene transfer approaches in plants have significantly improved. It seems that the safety of RPs and their potential for the inexpensive and large-scale pharmaceutically industrial production are important advantages of using transgenic plants as the green factories. However, their use raises some important concerns such as diffusion and amplification of transgene, contamination of food chain, buildup of environmental recombinant protein toxicity, and costs of subsequent processing. Therefore, further investigations are needed to produce valuable and therapeutic products using the safest, well-organized, cheapest and most efficient approaches.

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