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

Reza Mohammadinejad,[a] Amin Shavandi,[b] Diana S. Raie,[c] Jeyabalan Sangeetha,[d] Mohsen Soleimani,[e] Shahram Shokrian Hajibehzad,[f] Devarajan Thangadurai,[g] Ravichandra Hospet,[g] Jacob O. Popoola,[h] Ahmad Arzani,*[i] Miguel A. Gómez-Lim,[j] Siavash Iravani*[k] and Rajender S. Varma*[l]

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Plants have had numerous biological, clinical, pharmaceutical and medicinal purposes for many years; however, their use as a general platform for preparation of desired pharmaceutical and biomedical compounds is relatively current. Secondary metabolites with remarkable and diverse biological functions are produced by medicinal plants. Significant advancements in nanosciences have enabled their various applications in the development of a 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 is discussed.

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 and 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, but they do not have the capacity to produce the diversity of polymers synthesized by current chemical polymerization techniques. Nevertheless, by deploying the technology of gene transfer, it is feasible to define the compositions of the compounds of interest and have, in general, a control over their physicochemical properties and functionality, which is hard to achieve, using chemical techniques.[1]

The fields of biotechnology and gene-transfer have 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

[a]*Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran*
[b]*BioMatter unit-Biomass transformation Lab (BTL), École interfacultaire de Bioingénieurs (EIB), École polytechnique de Bruxelles, Université Libre de Bruxelles, Avenue F.D. Roosevelt, 50 - CP 165/61, 1050 Brussels, Belgium*
[c]*Egyptian Petroleum Research Institute (EPRI), Nasr City, Cairo, Egypt*
[d]*Department of Environmental Science, Central University of Kerala, Kasaragod 671316, Kerala, India*
[e]*Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[f]*Molecular Plant Physiology, Department of Biology, Faculty of Sciences, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[g]*Department of Botany, Karnatak University, Dharwad 580003, Karnataka, India*
[h]*Department of Biological Sciences, Biotechnology Centre, Covenant University, P.M.B. 1023, Canaanland, Ota, Ogun State, Nigeria*
[i]*Department of Agronomy and Plant Breeding, College of Agriculture, Isfahan University of Technology, Isfahan-8415683111, Iran. E-mail: a_arzani@cc.iut.ac.ir*
[j]*Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato, Km. 9.6 Libramiento Norte, Carretera Irapuato-León, 36824 Irapuato, Guanajuato, Mexico*
[k]*Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: siavashira@gmail.com*
[l]*Regional Centre of Advanced Technologies and Materials, Department of Physical Chemistry, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 27, 783 71 Olomouc, Czech Republic. E-mail: Varma.Rajender@epa.gov*

is for this reason modern systems deploy the transient expression of heterologous proteins that relies on viral vectors, which do not result in transgenic plants. Plants are an excellent resource for greener production of biomaterials and in this critical review the recent progress in the plant-based fabrication of biomaterials such as collagen, gelatin, polyhydroxyalkanoates (PHAs), silk and elastin has been highlighted.[2–5]

The application of phytomedicines has increased due to their therapeutic value when compared to allopathic medicines as these bio-compounds exhibit fewer side effects. A better understanding of the function and kinetics of phytopharmaceutics 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 used in redox reactions 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 a small particle size leads to the access to the whole surface area of the drugs and in turn enables rapid dissolution in the blood. In addition, drug delivery is targeted in a specific manner to a desired site of action. Due to their very minute size, microspheres and liposomes can easily pass *via* sinusoidal spaces in the bone marrow and spleen as compared to other systems. 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 drugs, 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 was diminished, when it was coupled with nano-silver (Ag) and intraperitoneally injected into rats.

Phytomedicines have become more popular for their potential use in curing many kinds of ailments with high therapeutic value and low 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 a 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 leads to enhancement in the stability, increase in solubility, development of pharmacological activity and sustained delivery, improvement in macrophage distribution and protection from chemical and physical damage.[14]

An innovative phyto-nanomedicine prepared *via* well-formulated routes of synthesis, by virtue of its size, increases the dissolution and bioavailability of drugs while reducing its dose. *Cuscuta chinensis*, containing lignin and flavonoids as active therapeutic compounds, shows poor aqueous stability and

Reza Mohammadinejad

Reza Mohammadinejad studied Biotechnology at the Graduate University of Advanced Technology, where he obtained a M.Sc. in 2013. In 2016 he started his Ph.D. in the Pharmaceutics Research Center at the Kerman University of Medical Sciences. Here, his research interests lie in the development of nanotheranostic systems and CRISPR gene editing therapies.

Ahmad Arzani

Ahmad Arzani is professor of Plant Genetic and Biotechnology at Isfahan University of Technology, Isfahan, Iran, where appointed as instructor and then awarded a PhD scholarship to pursue his study at the University of Sydney. Dr. Arzani has been Principal Investigator of several research projects in his area of expertise. He has spent four sabbatical leaves at Colorado State University (2001–2), Chicago University (summer 2011), University of California-Davis (2014–2015), and Northern Arizona University (summer 2018) as visiting professor. Dr. Arzani has published over 200 national and international journal articles, and over 30 full papers in the proceeding of international conferences. He has also several review articles and book chapters.

solubility upon oral administration. The nano-sized drugs from *C. chinensis* were produced by nano-suspension techniques due to their antioxidant and hepatoprotective effects. Similarly, *Radix salvia* NPs have been synthesized by the spray drying method for the treatment of coronary heart disease and myocardial infarction.[15,16] The potential utilization of nanotechnology techniques leads to an increased bioactivity and bioavailability of phytomedicine by minimizing the size of particles, surface modification, and entrapping the phytomedicine with various polymers of nanomaterials. In the future, it is necessary to focus on the design and development of new multifunctional novel nanomaterials and *in vivo* studies of their formulations for effective application in the pharmacological field.[15,16]

2. Phyto-nanotechnology and plant-made nanostructures

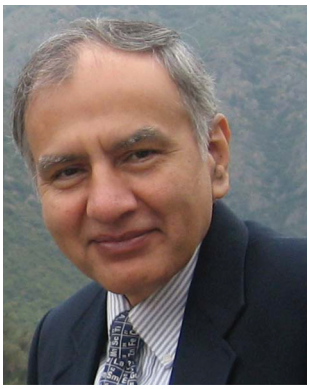
Compared to the traditional methods of nanoparticle synthesis using toxic and hazardous materials, plant-based eco-friendly and greener nano-approaches for the assembly of NPs are showing major advantages. Plant extracts are renewable in nature and often are processed in eco-friendly aqueous medium. Moreover, reaction conditions used in production processes are mild.[15–19] Additionally, plant extracts and phyto-nanoproducts are receiving consideration as they are cost effective, non-hazardous and energy efficient. Based on green chemistry principles, three main steps should contribute in the production of greener NPs: the selection of (i) 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 the phyto-nanotechnology arena because of the complexity of plant systems and other reproducibility limitations. Phyto-nanotechnology has great potential in the production of different NPs by 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 the preparation of novel pharmaceuticals, imaging, drug delivery, diagnosis methods and 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 of nanometer size *i.e.* 10^{-9} m.[25] Developing alternative eco-friendly methods for generating NPs is imperative.[26] During the last few decades, scientists have directed their investigations to the biosynthesis of nanomaterials as a 'bottom-up' track; numerous organisms could synthesize NPs in an ambient environment (pressure and temperature) avoiding the generation of dangerous agents and

Siavash Iravani

Dr. Iravani has worked on several academic research projects at the Isfahan University of Medical Sciences (Faculty of Pharmacy and Pharmaceutical Sciences), including green and eco-friendly synthesis of nanomaterials, plant-derived nanostructures, phytochemical analysis, graphene-based nanocomposites, water treatment technologies, nanoparticles for drug delivery in cancer, nanocarriers, and drug nanoparticles. His previous experience, of more than seven years, centers on drug development and industrial pharmacy in various capacities including research and development, formulation, and quality control. Dr. Iravani has authored over 50 peer-reviewed scientific publications including twelve book chapters and one book.



Rajender S. Varma

Prof. Varma (H-Index 104, Highly Cited Researchers 2016–18; Publons Awardee 2018) was born in India (Ph.D., Delhi University 1976). After postdoctoral research at Robert Robinson Laboratories, Liverpool, U.K., he was faculty member at Baylor College of Medicine and Sam Houston State University prior to joining the Sustainable Technology Division at the US Environmental Protection Agency in 1999 with appointment at Palacky University at Olomouc, Czech Republic. He has over 45 years of research experience in management of multidisciplinary technical programs ranging from natural products chemistry to development of more environmentally friendly synthetic methods using microwaves, ultrasound, etc. Lately, he is focused on greener approaches to assembly of nanomaterials and sustainable applications of magnetically retrievable nanocatalysts in benign media. He is a member of the editorial advisory board of several international journals, has published over 515 papers, and has been awarded 16 US Patents, 6 books, 26 book chapters and 3 encyclopedia contributions with 36 000 citations.

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 the 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 reported,[30] *via* the reduction of Ag nitrate,[31] including formation inside the plant cells.[32] Although a change in the 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 NP preparation with ground plant biomass and their characterization were demonstrated using alfalfa plant, *Medicago sativa*[34] with experimental proof of the synthesis in living vascular plants.[35] Several reports have shown the potential applications of various plant parts 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 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 the 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 NP 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-accumulator plants have the capability to accumulate meaningful metal concentrations in their cell wall, vacuole, and 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] have the capability to biosynthesize NPs inside their tissues.[35,49,51–53] Briefly, when seedlings of *M. sativa* were grown on potassium tetrachloroaurate, $KAuCl_4$,[35] and $AgNO_3$,[52] in a 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 shoots suggested that they actively transport Au atoms; the existence of Au NPs in the size range of 2 to 20 nm indicated the 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; the absorbed Ag^0 undergoes 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 5–50 nm diameter have been synthesized within *B. juncea* tissues grown on soil supplemented with $AuCl_4$, 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 plant metabolic pathways and specifically, the possible reduction of the above-mentioned metals using *M. sativa* and *Ipomoea lacunosa* has 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 materials.[46] Thus, plant-mediated Au NPs were synthesized hydroponically using seedlings of *S. drummondii* intracellularly grown in aq. $KAuCl_4$. 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 the 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 the 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 were 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 they were found also in vascular bundle tissues and cell walls in smaller amounts. From the nitrate source, spherical NPs were in the size range of 5–140 nm in the leaves, 40–60 nm in the stem, and 10–30 nm in the root. However, NPs prepared from the 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 reducing sugars, responsible for the fabrication of Ag NPs, are variable among the species increasing the improbability that only a single substance was responsible for this reduction.[50]

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* concentration 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 were 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 mass to their shoots than *M. sativa*.[47] Ag and Au salts were absorbed by *B. juncea* and transported in the plant either as metal nanoparticles 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 the 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 these metals.[66] Moreover, liquid culture-grown seedlings of *A. thaliana* (L.) upon 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 NP biosynthesis using living plants

In general, 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 plants.[56] The complicated redox control for physiological pathways in plants is restrained by an array of biomolecules such as 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 the size range 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 an 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 the ions of Ag into the metal nano-form wherein the exposure of living seedling and callus to 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 the callus, Ag^0 ions 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 mor-

phology; mono-dispersed spherical shaped NPs in the size range from 2 nm to 7.5 nm were bio-synthesized by seedlings at pH 10.[58] Under the same alkaline conditions, *i.e.* pH 10, the size of NPs which were bio-generated by the 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 the cell surface and the cell wall. Hence, ions were reduced into metals due to the transmembrane dehydrogenases/reductases of the 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 they 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 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 the size range 5–50 nm.

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

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

Generally, metallophytes or hyper-accumulators can take up 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*, cadmium (Cd)-dependent modifications in beta carbonic anhydrase initiate the photorespiration improvement which might protect the photosystem from oxidation.[73] The adjustments produced by Cd disrupt the alleviating communication 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 the electron transport machinery of the cell, culminating in excess production of ROS that damage the macromolecules and thus creating oxidative stress (OS) within the cell. Moreover, the plant hormones 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 death. These parameters instigate the 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 plants against ROS in heavy metal stress. Toxic MIs (at the cellular level) induce OS *via* generating ROS by promoting DNA impairment and/or weaken the DNA repair mechanisms, hamper the membrane functional reliability and 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, the 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 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 their native conformations were degraded *via* the ubiquitin proteasome process, called 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 involvement 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] Furthermore, 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, and sequestration and trafficking into the vacuole *via* vacuolar transporters.[80] Phytochelatins (small cysteine-rich oligomers) produced at the initial stages of metal stress have 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 the phytochelatin synthase (PCS) gene did not

always bring about an increased tolerance to heavy metal stress in plants. It has been demonstrated that phytochelatin production was enhanced by 2.1-fold, when compared to wild-type plants.[83] Furthermore, additional phytochelatin 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 the cytosol to the vacuole for appropriation *via* transporters. In plants, vacuolar confiscation is an important mechanism for 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 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 MT-deficient mutants accumulated 30% and 45% less Cu in roots and shoots, 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 MT 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 the metal–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 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 promoting discerning degradation of misfolded or denatured proteins or circumventing protein aggregation.[71,94]

By using proteomics, researchers can recognize the operative genes or proteins participating in the reactions of plants to heavy metal stress at the molecular level.[95] Transcript investigations of several plant species demonstrated that HSP70 was highly expressed at varied metal stress levels.[96] HSP70 chaperones, alongside their co-chaperones such as DnaJ, render a group of leading cellular machines to avert the accumulation of freshly produced proteins as aggregates and safeguard the proper folding of proteins 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 protein-induced 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 unpredictable environments. Moreover, in another study, Xu *et al.*[101] reported that heterologous expression of AtBiP2 protein in BY-2 acted as a damage regulator of Cd-induced ER stress and programmed cell death. Furthermore, Guan *et al.*[102] reported that ER chaperone binding protein operated as a positive regulator in the Cd stress tolerance scenario. The transcription level of the glutathione (GSH) gene was examined in LcBiP-overexpressed tobacco to examine the mechanism.[78] Liu *et al.*[103] reported a plant-specific component of the 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 mechanism, whose mutation subverts AtHrd1a to relegate polyubiquitination. Furthermore, 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 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 the regulation of plant heavy metal stress tolerance.[107] The genome-wide transcription investigation of rice plants demonstrated that smaller concentrations of Cd conducted the induced polyubiquitin expression in shoots and roots.[108] Under extreme situations, over-expression of genes occurred in the ubiquitin proteasome process cascade, thus enhancing tolerance to manifold stresses without any undesirable effects on the development and growth in plants.[109] The heterogeneous expression of the rice E3 ligase enzyme synthesis RING domain OsHIR1 gene in Arabidopsis was reported to be reduced with the buildup of As and Cd in both shoots and roots.[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) revealed that among the 30 ATGs genes, 18 ATGs genes were up-regulated by more than two folds through at least 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]

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 tools can be used to overwhelm the bioactive-compounds availability limitations from medicinal plants and to improve the productivity beneficial from 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 microbe cells cultured in large-scale bioreactors.[4,114] Gene transfer technologies such as *Agrobacterium tumefaciens* mediated transformation or particle bombardment (physical delivery) have been deployed to generate stably transformed plant or transient expression. Thus using plants for the preparation of recombinant non-pharmaceutical and pharmaceutical proteins, and 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 in eukaryotic post-translational machinery function, (iii) lower risks of contamination from animal pathogens, and (iv) being amenable to the large scale manufacturing process (Fig. 1).[117]

Tissue-restrictive promoters can be applied for the production of RPs *via* selective expression of therapeutic genes in the 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 cellular

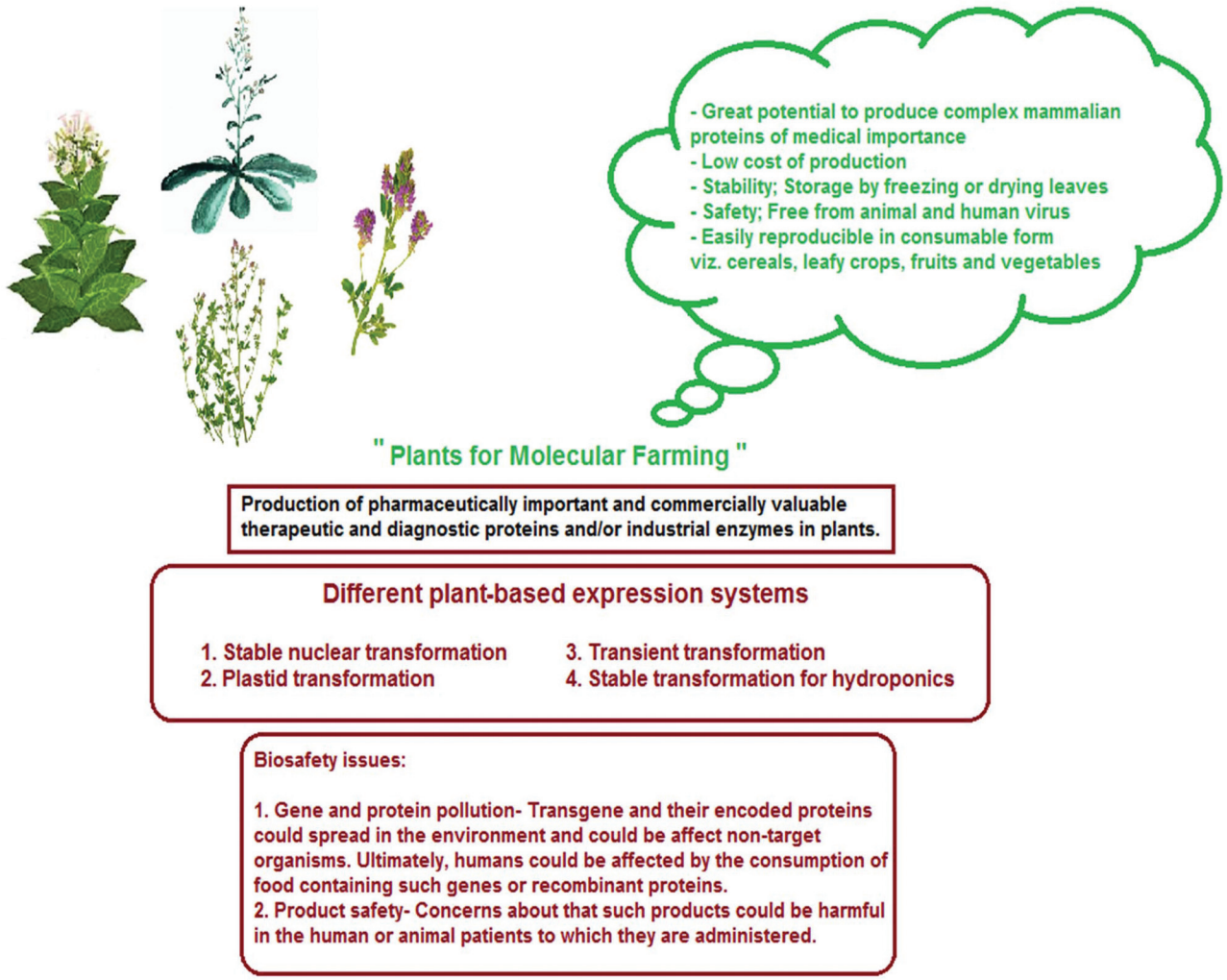


Fig. 1 Plants for molecular farming: advantages and biosafety issues.

compartments such as the plastids, apoplast, cytosol or endomembrane lumen by particular peptide tags. The ER, the first organelle in the secretory pathway, is transmitted by the plasma membrane, vacuolar, lysosomal and secretory proteins, and exports proteins to the Golgi apparatus for sorting to their final destination after ensuring the correct folding and assembly of polypeptides.[119] Secretory proteins, 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 the whole seed expression promoter, aleuronic layer-specific promoter, endosperm specific promoter (predominant expression in the whole endosperm subaleurone layer, the inner starchy endosperm zone) and transfer cell specific- and embryo expression-promoter. The plant cell and its organelles, seed anatomy and protein targeting organs can be fully exploited to accumulate a 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) the cytosol, apoplast, vacuole, and chloroplast can be used to target the RPs which can also be maintained in the ER. Targeting plastids results 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 the 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), *Oryza sativa* (rice), and *Triticum aestivum* (wheat).[121] Plants can be genetically modified in order to produce chemicals and pharmaceuticals for biomaterial purposes. The advent of plant molecular farming (PMF) had re-invigorated and elicited global interest 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 the present chemical polymerization techniques. Nevertheless, using the gene transfer technology, it is possible to determine the molecular weight and 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 fields of biotechnology and gene transfer have helped in circumventing the limiting factors. Transgenic plant 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 the 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 genes *via* viral vectors or introduction to nuclei and chloroplast genomes.[127] This technique can be 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 has soared apparently due to an increase in demand and the risks associated with animal derived biomaterials such as contamination with pathogens and enhanced disease transmission risks.[129] Other factors that make plant-derived substitutes in high demand include safety, being 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 protein quality and the presence of impurities in the product are some of the major drawbacks of PMF[131] which have hindered the approval issues and therefore a wider distribution and usage in the pharmaceutical and food industries.[131,132]

Collagen represents 30–40% of the protein in the body and comprises 90% type I collagen and non-collagenous proteins like glycosaminoglycans. It accounts for 90% of the inorganic phase of the bone matrix.[133] A collagen molecule has three compartments of α chains which form a triple helix structure; this alpha chain consisted of repeating sequences of Gly-X-Y where X is proline and Y is hydroxyproline residue.[134] The correct formation of the collagen triple helix depends on the presence of all these sequences. The network consists of well-organized parallel fibers or bundles. The collagen fiber is composed of long tough molecules known as tropocollagen comprising various peptide chains. Based on the amino acid sequence of tropocollagen different collagens 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 consists 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, is an integral part of extracellular matrix (ECM) tissue engineering with proven advantages for repair and restoration of the injured tissue,[134,137] 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, disk, pellet, NP and tablet forms.[139] Ever since 1981, bovine-derived collagen has been in use in the biomedical industry; it has been a standard to which all other soft tissue reinforcement materials are related to. However, over 35 years later, it is nonetheless not the ideal material because it is costly and can induce allergic reaction in a tiny percentage of patients.[140] Collagen is commercially extracted from animal hides (porcine skin, bovine tendon)[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 be treated with anti-allergic drugs such as cyclosporine.[140]

Despite these reactions which are normally resolved in due course, other serious drawbacks such as incomplete adsorption and implant functional failure have also been 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 limits the collagen application in an emergency situation.[143] Apart from the hypersensitivity reactions and the time consuming process of the skin test, the transmission risk of prions to human cells is another issue in the 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 has 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 affect the 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 its 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 an accelerating requirement for collagen-based biomaterials has paved the 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] Fig. 2 shows a schematic overview of the production of recombinant collagen using transgenic tobacco leaves. Nonetheless, hydroxylated collagen can be produced in a relatively large quantity using transgenic plants.[147] In early studies, Ghosh *et al.*[151] reported a 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 human-derived collagen; vacuoles were targeted for expression of procollagen that consequently resulted in the formation of thermally stable and dense packed mature collagen with a triple helix structure.[141] The alpha helical structure was preserved and the extracted collagen showed the characteristic properties of tissue-derived collagen such as a high surface area, binding sites and a high water holding capacity. Plant-derived collagen showed comparable properties in terms of processability and pharmaceutical application and efficiency.[148]

Willard *et al.*[130] compared plant-derived human collagen (PDHC) and bovine derived collagen (BDC) using electrospinning and freeze drying processes 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 PDHC were thinner and more round and uniform. Furthermore, PDHC showed superior solubility in acetic acid and better biocompatibility

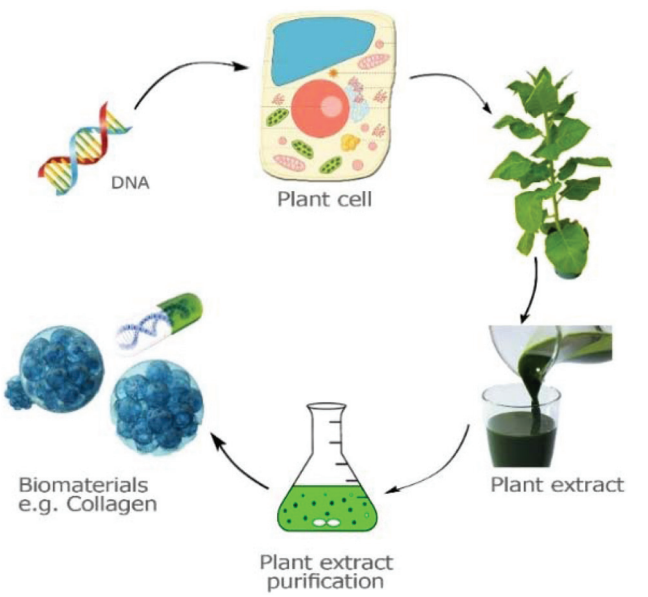


Fig. 2 Schematic overview of production of recombinant collagen using transgenic tobacco leaves.

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 the higher viscosity and higher collagen content of the bovine sample at the same concentration (80% *vs*. 97%), and therefore a scaffold with a 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] are much lower than 2.7–10 Mpa[155] for human skin. Therefore, a technique such as using high strength polymers in the production of biocomposites[154] is currently researched to enhance the mechanical characteristics of the fiber. The wound healing properties of PDHC (Vergenix FG) were assessed by Shilo *et al*.,[156] using pigs and rats as big and small animal models and they found a faster healing process in the animals treated with PDHC. It has been reported that wounds shrunk after 24 hours, and a 66% closure was recorded after 6 days in the rat model while in the porcine model 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. The authors concluded that the yield was significant compared to 10–100 mg (ref. 157) that was obtained *via* targeting the nucleus in plants. Tobacco plants have been used for many years for production of plant-derived proteins, because this plant can be easily regenerated in tissue culture within 6–8 weeks and is readily amenable to transformation. Tobacco has now gained a well-established plant host status for the expression of proteins.[3,158] It provides high yields of biomass, is rapidly scalable and also is not considered as a food or feed crop and therefore, it 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 humans based on DNA and/or protein sequence information.[159] Ruggiero *et al*.[160] transformed tobacco plants using cDNA to encode the human preproα1 chain for collagen I, and suggested that the triple helix of collagen can be produced; although the final product was completely transformed to collagen, the product had lower thermal stability due to the lack of prolyl-hydroxylation in the plant. Hydroxylation of proline residue in the sequence Xaa-Pro-Gly of the helical structure of collagen is required for proper functionality of the ECM in the 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 the 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 a higher percentage of (Hyp) (18%) *via* co-expression with human P4H; co-expression of rP4H increases the thermal stability of rCIa1 with the reported yield of up to 12 mg kg^{-1}.[161] The 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	30 mg kg^{-1} dried leaf	0.53	160
Tobacco	N/A	N/A	164
Tobacco	0.5–1 mg kg^{-1} leaf		162
Tobacco	200 mg kg^{-1} fresh leaf	7.55	165
Barley P1 cell	2–9 μg per cell culture	NA	166
Barley seed	Below detection	2.8	167
Maize seed	200 mg kg^{-1} seed	2.01	168, 169
Maize seed	3 mg kg^{-1}	1.23	169
Maize seed	15.9 mg kg^{-1} germ (CIα1) 49.6	NA	169
Maize seed	12 mg kg^{-1} seed (CIα1) 4 mg kg^{-1} seed (CIα1-OH)	18.11	161
Tobacco plants	2 percent of the extracted total soluble proteins	NA	130
Tobacco plants	1 g kg^{-1} 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, 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 that has an important role in attachment, growth and proliferation of the cells, by providing a suitable substrate for different cells to attach to and grow. It has been reported that recombinant gelatin 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 and store a 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^{-1} 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] A 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 bandages, intelligent drug delivery and absorbents.[125,172] Expression in *E. coli* of a manmade protein comprising 121 repeats of the Gly-Val-Gly-Val-Pro peptide was demonstrated to culminate in the buildup of the polymer in inclusion bodies.[173,174] However, only low polymer accumulation was obtained when the same expression was performed in the fungi *Aspergillus nidulans*;[175] the reasons for these lower accumulations are not clear but the chloroplast is the predominant location within cells 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 an elastic biopolymer in tobacco with the amino acid sequence of GVGVP and showed that using chloroplast for the expression is 100 fold more efficient compared to nuclei for the production of elastic biopolymers.[172] A 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 and 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, 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 collagen with comparable properties to animal-derived collagen. Therefore, until these issues are 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 animals is difficult in larger quantities; there are some active companies such as Collplant (http://www.collplant.com) that have some plant-derived collagen products on the market but the products do not have approval for sale in the European or the US market. This niche field might be applied as the experimental base that might help to develop the technology and get plant-based strategies close to large scale production and possible clinical application.[148]

4.4. Production of recombinant anti-cancer monoclonal antibodies

Even though artificial production of antibodies by means of mammalian cell culture systems has acquired a great success in neutralizing a wide-range of diseases, a high level of production costs together with a long period of manufacturing time has challenged their scalability and utility.[178] Plants do not produce antibodies against their viral, microbial and fungal enemies. Naturally, antibodies are produced by human and other mammal immune systems. 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, plant-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 compared to typical mammalian serum-type immunoglobulin G (IgG).[180] For instance, IgG could be expressed only by introducing two foreign genes into 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 about 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 the heavy and light chains of typical IgG and monitored for polypeptide expression. Furthermore, the two separate lines were 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 reconfirmed 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 systems biology procedures such as diagnostics, reagents and therapeutics.[183–185] It appears that applying PMF as a rapid and scalable technology along with these kinds of advanced techniques will, there-

fore, help humans to overrule future challenges. Three kinds of applications of 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 seem to be the most promising.[186] As the second application, it has been demonstrated that plants can be engineered to produce predesigned antibodies for agricultural applications like to employ as fungicides and bactericides. In this manner, synthetic plants are applied to produce AOI to act as the plant artificial immune system against enemies like pests, bacteria, and viruses. The purpose of this technique is to produce synthetic eco-friendly pathogen-resistant 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, and destroy a 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 revived by overcoming some technical obstacles affecting antibody production in plants.[190]

Various kinds of materials such as synthetic particles (*e.g.*, polymers, gold and lipids) and biological materials (*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 various ranges 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 the 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 a 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) were briefer than those (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 leaf 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 the obtained mAbs CO17-1A from 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, the mechanical disruption method, buffer composition, *etc*.[196] In this study, three various lines of transgenic plants were investigated to evaluate the parameters affecting 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 mainly a critical consideration.[196] It has been reported that the optimal extraction method might be changed with the IgG target location in plant cells, the antibody yield dependence on the employed physical extraction approach, and the pH of the extraction buffer and the temperature of 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, the influence of the extraction buffer pH was evaluated in transgenic plants. Three leaf discs were ground in buffer of 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 a pH of about 5 to 7 was appropriate. When the extraction buffer approached the pI value of IgG1 (8–9.5), less monoclonal antibody 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 the production of antibodies lies within a combination of both low level of expression and high level of proteolytic degradation which results as loss of the end-product[199] (Fig. 3). Low level of expression is not a specific matter for antibody production in plants because it is found in other recombinant proteins. There have been a large number of attempts to efficiently increase the expression level; the use of the chloroplast genome instead of the nuclear genome has been shown to incredibly boost the expression level.[200] In other attempts, the crucial role of expression cassette elements in antibody expression like codon optimization,[201] viral suppressors of RNA silencing (VSRs),[202] and innovative enhancing insulators have been ascertained.[203]

The production of truncated antibodies with additional and/or smaller than expected fragments has been reported in different tissues such as leaf, seed, callus, and tuber of various plant species.[204] This truncation could be because of both mis-assembling which is the final step in the antibody production procedure and the consequence of extracellular protease 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 dependent on the host–antibody interaction, it is not easy, even though a lot of attempts have been 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 most

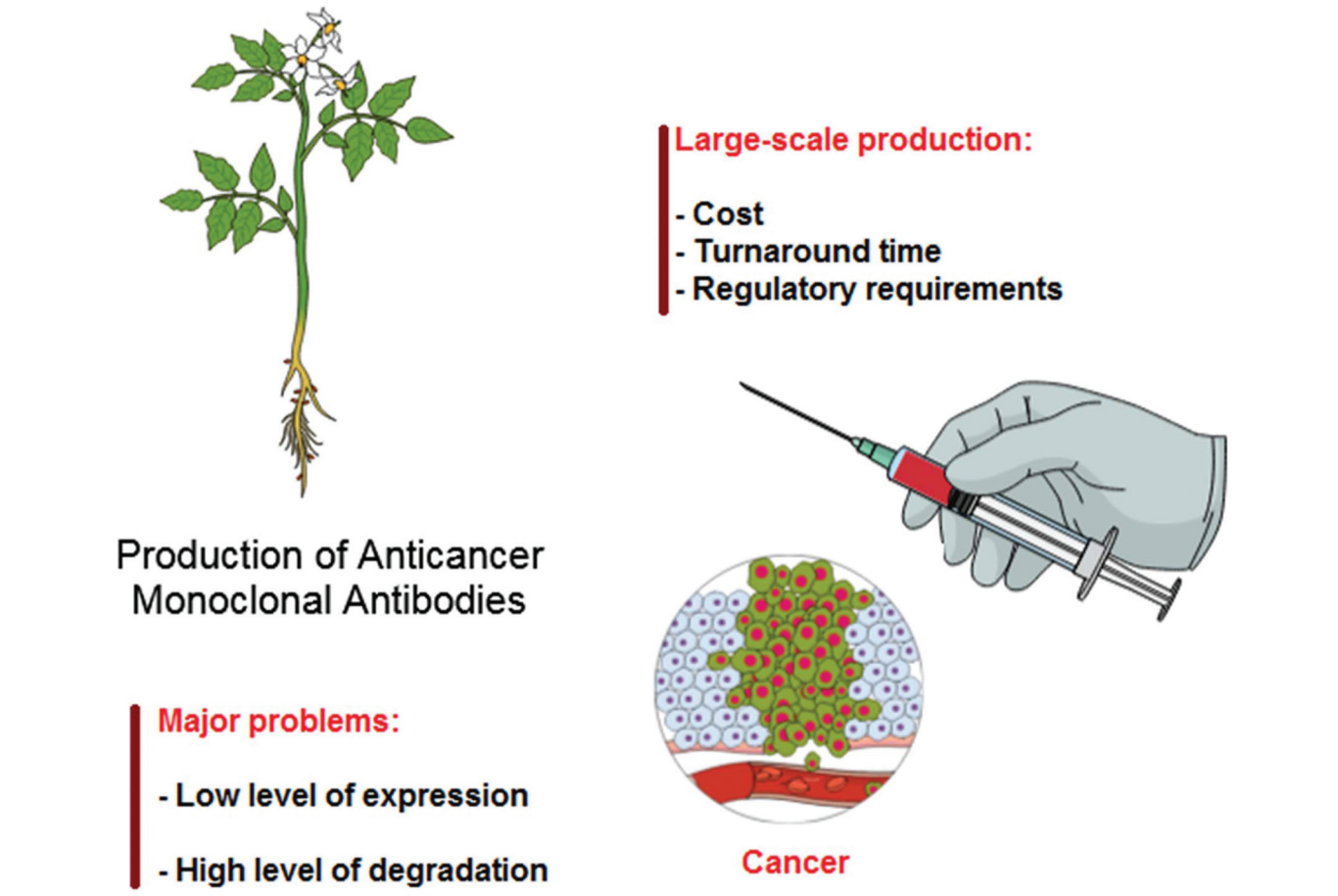


Fig. 3 PMF and production of recombinant anticancer monoclonal antibodies.

likely occurred during purification possesses in extracellular medium such as apoplast or during secretion from the ER to the 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 the ER. The ER has been long accepted as a safe place for heterologous protein accumulation which can easily be derived by adding the KDEL signal peptide that drives the proteins of interest into the ER.[205] Furthermore, by co-expressing protease inhibitors with the antibody of interest and targeting them into the apoplast, scientists 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 assessment. These vaccines can be produced on a large scale. Tissue positions, plant growth conditions (such as temperature, drought stress, multi-dimensional 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 is 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 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 vaccine 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 the 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 the healthy populace to prevent cancer from occurring. Some important advantages of anticancer vaccine production using plant systems and related critical issues are highlighted (Fig. 4).

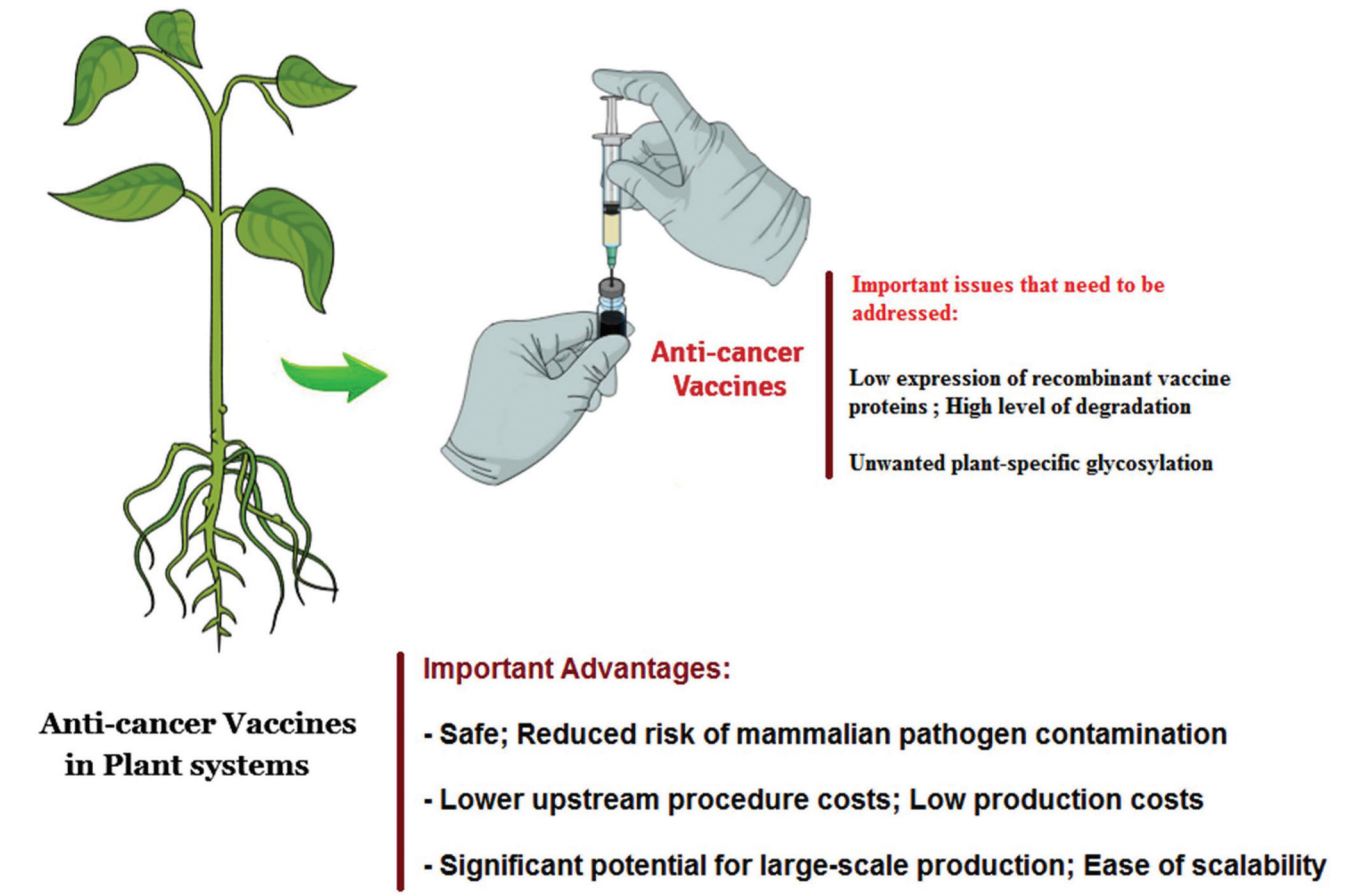


Fig. 4 Anticancer vaccine production using plant systems: advantages and some important issues.

Anti-cancer vaccines generated in plants might be positioned for 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 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]

One of the important candidates for anticancer vaccine production is E7 oncoprotein from Human Papilloma Virus (HPV). Massa *et al*. reported the HPV16 E7 coding sequence (wild type or mutagenized sequence, E7GGG) as a fusion to β-1,3-1,4-glucanase (LicKM) of *Clostridium thermocellum* and it was 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 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 a plant-based biomaterial depends on its sufficient extraction at the required level in a sustainable yet efficient and economical way. Despite the number of different studies that explored various means of extracting protein polymers from plants, 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 the not too distant future, molecular farming will see substantial accomplishments with precise and technical inquiries; significant concerns are summarized in Fig. 5. Basic and original investigations are required for the commercial success of ensuing products in spite of some advances that have occurred in the 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) has 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

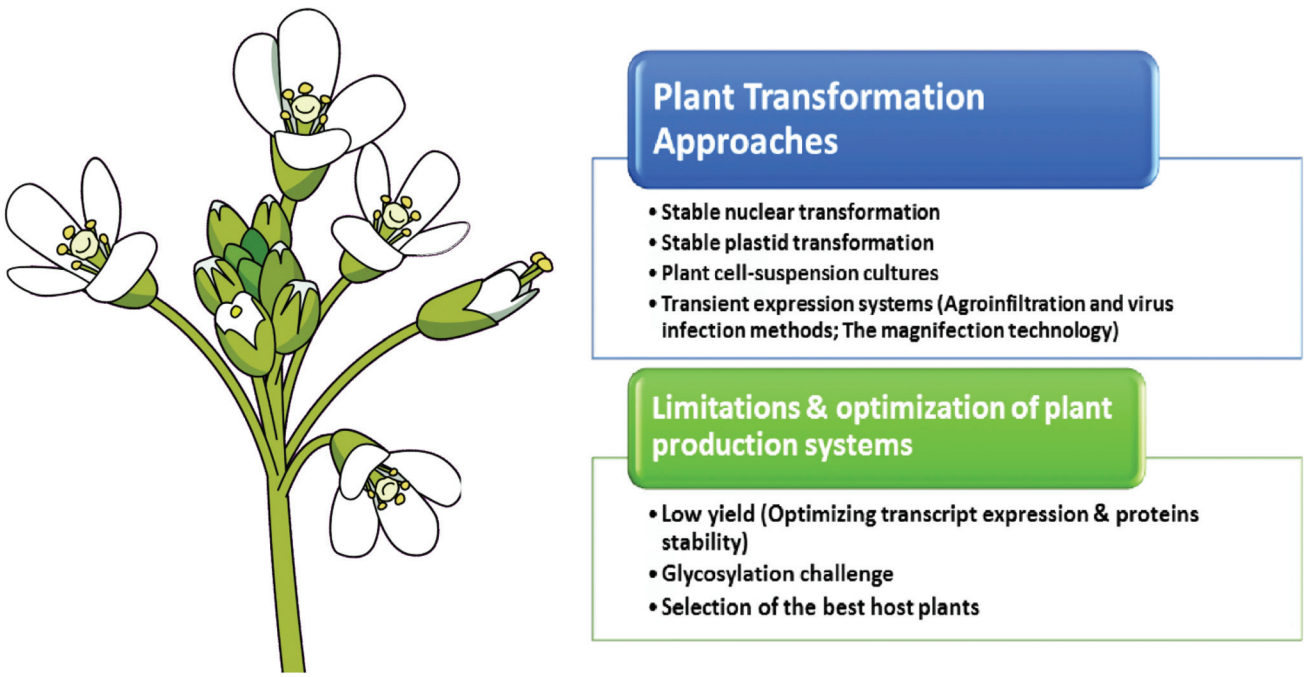


Fig. 5 Plant molecular farming and related significant concerns.

comprehensively the extraction and downstream processing of plant-derived recombinant proteins.[214]

In the 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

Plant molecular farming is the generation of therapeutically significant and commercially viable secondary metabolites and RPs in plants, and its success is dependent on the genetic transformation of plants attained by 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 recombinant proteins and their potential for inexpensive and large-scale pharmaceutical industrial production are important advantages of using transgenic plants as green factories. However, their use raises some important concerns such as diffusion and amplification of transgene, contamination of the 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.

Author contributions

AS, DSR, JS, MS, SSH, DT, and RH prepared the initial draft of the article, JOP, AA, and MAG organized the draft and revised its contents, and RM, SI and RSV did the drafting, organized the draft and revised its contents, revision of the main intellectual contents and the approval of the final version.

Conflicts of interest

There are no conflicts to declare.

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