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Tobacco as a Transient Therapeutic Peptide Bioreactor: A Fast and Efficient Drug Production Platform

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Abstract

In recent years, higher plants have been used as reactors capable of producing biologically active proteins, antigens used as vaccines and recombinant peptides (AMPs) with antimicrobial potential. Compared to other systems, plants have relatively low production costs and can make post-translational modifications necessary for some MPAs to present their correct conformation and be biologically active. In this context, tobacco plants are becoming important vehicles for the massive and transient biosynthesis of antimicrobial peptides and other therapeutic molecules. Magnifection™ technology enables the rapid production of large-scale therapeutic molecules in tobacco at relatively low production costs and is a very interesting system for the manufacture of drugs against diseases of economic and social importance. Recombinant and transient biosynthesis of AMPs in tobacco may be a viable alternative to complement the fight against pathogenic microorganisms that cause severe infections.

Keywords: Tobacco; Transient expression of genes; Magnifection™; Tobacco mosaic virus; Total soluble protein; Plant-made pharmaceuticals; Threonine; Deoxyribonucleic acid; Post-transcriptional gene silencing; Coding sequence

Abbrevations: AMP: Antimicrobial peptides; TMV: Tobacco Mosaic Virus; Ti: Tumor Inducing; TSP: Total Soluble Protein; PMPs: Plant-Made Pharmaceuticals; hGH: Human Growth Hormone; SAH: Human Serum Albumin; Ans: Asparagine; Ser: Serine; Thr: Threonine; DNA: Deoxyribonucleic Acid; PTGS: Post-Transcriptional Gene Silencing; CDS: Coding Sequence

Introduction

Currently, microbial resistance is one of the biggest threats to public health worldwide [1]. 25,000 people die each year in the European Union from infections caused by multi-resistant bacteria. In the United States, complications caused by hospital-acquired infections account for over 60,000 deaths per year [2]. The UK government estimates that by 2050 worldwide, the death rate will reach 10 million each year and the costs associated with treating these infections will total about $100trillion [3]. In Brazil, the number of carbapenemase-producing *Klebsiella pneumoniae* (KPC) strains has increased in recent decades and currently represents 96.2% of the mechanisms of β-lactam resistance in this bacterial species [4]. However, microbial resistance is not a problem that is restricted to the hospital environment only. The presence of extended-spectrum β-lactamase-producing *Escherichia coli* strains (CTX-M) has been demonstrated in wild fish off the Brazilian coast [5]. Multiresistant *Pseudomonas aeruginosa* strains were detected at a hospital effluent treatment plant in the state of Rio de Janeiro [6].

Thus, to fight resistant microorganisms, new therapeutic strategies need to be developed [7]. One may be the use of antimicrobial peptides (AMPs) as an alternative to antibiotics conventionally used to treat resistant strains [8]. However, some limitations that need to be remedied, such as the low level of production of these molecules by organisms. Thus, one strategy for producing AMPs at high concentrations is the heterologous expression of the genes encoding the peptides of interest in systems that function as "bio factories" [9]. Bacteria, yeast, and plants are the main systems used for recombinant protein and AMPs biosynthesis [9]. The bacterium *E. coli* is the most used prokaryote system as bio factory of therapeutic molecules [10]. The main attributes of bacteria as fac-

tories of drugs are the rapid growth rate, the high amount of commercially available vectors for bacteria transformation, well-established DNA manipulation protocols, as well as the availability of information about bacteria genetics, biochemistry and physiology [11]. Besides, the possibility of continuous fermentation and relatively low production costs contributes to the choice of this system for the recombinant production of drugs [12].

However, prokaryotic cell-based expression systems have some limitations when compared to other biological systems. Cationic AMPs are more prone to proteolytic degradation in bacterial systems [13], and this phenomenon can be circumvented by fusing the AMP to a much larger fusion protein that, after biosynthesis, can be chemically or enzymatically removed. However, this process increases one step in the purification of the molecule of interest what may increase production costs and decrease production yield [14]. Also, *E. coli* produces insoluble protein structures called "inclusion bodies" [12], which also may hamper AMP purification. Another limiting factor is that prokaryotic cells are unable to perform some essential post-translational modifications [9]. For these cases, it is necessary to use other platforms capable of doing them. Yeasts are eukaryotic organisms capable of circumventing some of the limitations presented by the prokaryotic system. Among them, *Saccharomyces cerevisiae* is widely used as a model organism because its biochemistry, genetics and cell biology are well known, as well as these organisms can produce complex proteins that can be used as vaccines, pharmaceuticals or as diagnostic reagents [12]. Although yeasts may perform posttranslational modifications such as glycosylation, their disadvantages are frequently related to protein hyperglycosylation [15].

In recent decades, *Pichia pastoris* yeast has been widely used for heterologous peptide and protein production because it is capable of performing disulfide bridges, O-glycosylation, N-glycosylation and signal sequence processing correctly [10]. Unlike *S. cerevisiae*, *P. pastoris* is not a fermentative microorganism, which allows its cultivation under a high cell density [9]. *P. pastoris* is a methylotrophic microorganism, i.e, it can use methanol as a carbon source [16]. However, when compared with *E. coli* and *S. cerevisiae*, this system does not have many commercial vectors, which constitutes a disadvantage in its use [9]. Recently, plants are also being used for the expression of transgenes of pharmacological interest, as they have advantages over other systems that use microbial or animal cells, regarding biomass accumulation [12]. Transgenic plants offer unparalleled scaling potential, enabling the cultivation of a large number of plants for pharmaceutical use. This capacity could be exploited, for example, to meet Southwest Asia's annual demand for the hepatitis B virus vaccine, which is estimated to need approximately 101 hectares (greenhouse space) to the cultivation of transgenic potatoes [17].

Plant molecular farming

The term plant molecular farming has been used to describe the use of genetic engineering in plant systems for the production of recombinant biopharmaceuticals, functional proteins, enzymes of industrial interest and secondary metabolites and therapeutic AMPs [18,19]. Therefore, both AMPs and larger biopharmaceuticals recombinantly synthesized in transgenic plants are referred to as PMPs (Plant-made pharmaceuticals), which can be used as vaccines or in the diagnosis and treatment of a wide variety of diseases [20,21]. In 1986, human growth hormone (hGH) was the first pharmacologically relevant protein to be produced in transgenic tobacco (*Nicotiana tabacum* var. Petit havana SR1) and sunflower (*Helianthus annuus*) plants [22,23]. Four years later, human serum albumin (SAH) was produced in transgenic tobacco and tomato plants [24]. Since then, several proteins (including antibodies, antigens, molecular transporters, and enzymes) have been recombinantly produced in different plant systems [21]. Besides, some recombinant proteins derived from plants have been marketed or are in advanced stages of clinical testing [21].

Currently, plant genome manipulation represents an unprecedented opportunity to produce affordable drugs with a global distribution reach [25]. When compared to heterologous expression systems based on bacterial cells, fungi or transgenic animals, some plant species present intrinsic advantages mainly due to the relative ease in genetic manipulation and lower production costs involved [18,26]. In this economic context, plants only need simple elements such as light, minerals, and water to produce abundant biomass, which is responsible for the accumulation of significant amounts of recombinant molecules in leaves, tubers, stems, and seeds [20]. Added to the low cost of production, plant systems also have as advantages the absence in the final product of pathogens common to humans, oncogenes and endotoxins, which reduces the additional expenses for purification, as well as not hyperglycosylating recombinant proteins [26,27]. Plants are also capable of correctly folding and assembling complex glycoproteins, such as antibodies, due to the presence of chaperone proteins homologous to those of animals [20,28]. Another advantage is that plants produce protein disulfide isomerase (PDI), which is responsible for catalyzing the formation of disulfide bonds, essential for the activity of certain antimicrobial peptides [29].

Most mammalian proteins undergo post-translational modifications after synthesis including proteolytic cleavage, phosphorylation, carboxylation, hydroxylation, acetylation, and glycosylation. Many, but not all, of these modifications, also occur in plants [30]. However, there may be differences such as the pattern of N-glycosylation of plant systems that differs slightly from that found in animals [31]. Plant N-glycans have mannose, β-1,2-xylose and α-1,3-fucose, which are not present in animals, thus presenting an immunogenic potential [18,28]. Therefore, for the production of "humanized" proteins (i.e. proteins that have a similar glycosylation pattern to humans) in plants, it is necessary to inhibit the addition of plant-specific glycans [32]. This can be done by knocking out the genes of the β-1,2-xylosyltransferase and α-1,3-fucosyltransferase enzymes, as well as producing recombinant human β-1,4-galactosyltransferase [33]. Another strategy is to use purified β-1,4-galactosyltransferase and sialyl transferase for *in*

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vitro modification of recombinant plant-derived proteins [26]. Inactivation of N-glycosylation sites in asparagine (Ans), serine (Ser) or threonine (Thr) residues, while not "humanizing" proteins, can also prevent unwanted glycosylation [34]. There are currently several approaches to heterologous protein production in plant systems [32]. These approaches consist of nuclear or plastid transformation of whole plants or plant cell suspension culture, which may express the transgene stably or transiently [31]. However, all these strategies can be classified as belonging to one of the following groups: stable recombinant biosynthesis or transient recombinant biosynthesis.

Stable recombinant biosynthesis

Recombinant protein production in transgenic plants can be termed as "stable type recombinant biosynthesis". This occurs from the integration of exogenous genes in the nuclear genome or chloroplast circular DNA of plants [30,35]. Plants can be transformed by the soil bacterium *Agrobacterium tumefaciens* carrying the transgene, from the biolistic technique, in which plant cells are bombarded with particles containing the adsorbed exogenous DNA, or by electroplating protoplasts [27]. The transgene will be integrated into the plant genome (nuclear or chloroplastidial), which implies, in most cases, its transmission to future generations [36].

Nuclear transformation is still a widely used method for heterologous expression of transgenes in plants and is responsible for the products available on the market today. The main advantage of this approach is the accumulation of recombinant proteins and peptides in the seeds. These can be harvested dry and stored without the need for special packaging, reducing additional costs. On the other hand, transgenic crops can cross with native species, which is a disadvantage of the method, because the containment of transgenes is necessary [31]. A viable alternative to nuclear transformation may be a transplastomic expression, i.e. the production of recombinant proteins within plant chloroplasts [37].

This is because the chloroplast genome is inherited from the maternal genetic line, which eliminates the risk of transgene spread during pollination [36]. However, although they are not capable of glycosylation, it is possible to produce functional recombinant proteins in these organelles, as they allow correct folding, as well as the formation of disulfide bridges [19]. The advantages of plastids are still the absence of gene silencing, transgene stability over generations, accumulation of recombinant proteins between 4% to 31% of total soluble proteins (TSP), due to a large number of plastids in a single cell, and the ability to express polycistronic units [38]. Although stable recombinant biosynthesis was widely used in the early days of Molecular Farming, the use of a stable expression has been decreasing in recent years [39]. In large part, this is because this technique requires a long period for the development of a transgenic plant [32], thus leading to the search for other efficient strategies. One is transient recombinant biosynthesis, which is already being widely used for recombinant protein production [40].

Transient Recombinant Biosynthesis

Unlike the previous approach, in transient recombinant biosynthesis, heterologous proteins are produced without stable transgene integration in the plant genome and its vertical transfer [39,41-44]. Gene expression, which may or may not be extrachromosomal, begins within the first 24 hours after transfection and continues for several days or even several weeks, depending on the vector used and the protein in question [40]. Transient heterologous expression can be done through three different approaches: biolistic, infiltration of plant tissues with recombinant *Agrobacterium tumefaciens* cells, a process known as "agroinfiltration" or from plant infection with modified viral vectors [35,45]. Agroinfiltration is the most used technique for the transfer of transgenes to plant cells in transient expression [42]. When compared to stable transient recombinant biosynthesis is advantageous because it is a fast, relatively simple and efficient method [46].

The presence of RNA molecules in abundance for long periods in plant cells can trigger post-transcriptional gene silencing (PTGS). Thus, the use of transient recombinant biosynthesis can inhibit these mechanisms by producing a high amount of recombinant protein in a short period [38]. This feature makes this system ideal for obtaining products with uneven demand and unstable markets [29]. Another advantage of transient heterologous expression system is the ability to express, depending on the case, massively several transgenes at the same time for the assembly of multimeric protein complexes [40].

The Magnifecton™ Technology: massive and fast biosynthesis of therapeutic molecules in tobacco

In the early 2000s, Gleba and colleagues developed an efficient system for the transient expression of transgenes in tobacco plant cells called Magnifection™ [47]. The main advantages presented by this method are:

a. the absence of introgression, since the transformation of plants occurs without the transgene integration into the germ cell genome;

b. the rapid obtention of recombinant proteins and AMPs, usually occurring within seven days of infiltration; and

c. the ability to produce heterologous molecules at high concentration [21], in some cases heterologous production may reach levels of up to 80% of TSP [48].

This system takes advantage of the ability of the bacterium *A. tumefaciens* to transfer the T region of its Ti (tumor inducing) plasmid to the plant cell genome plus modified viral vectors derived from the tobacco mosaic virus (TMV). These vectors also have potent viral promoters that regulate transgene expression and do not require host cell enzymatic machinery to perform functions that are unnecessary for heterologous expressions, such as biosynthesis of viral coat proteins and viral motility proteins [42]. Thus, agroinfiltration of tobacco leaves cells allows the massive heterologous expression of genes that are cloned in the Ti plas-

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mid, taking full advantage of the production potential offered by the large plant biomass [49].

For the transformation of plant cells, three deconstructed viral vectors are used, that is, vectors that have been excised or truncated virulence genes, which are called pro-vectors. Two of them contain the separate viral genome, which has been optimized by introducing numerous plant introns, point mutations, and removing critical splicing sites. The third pro-vector carries the coding sequence (CDS) of a bacteriophage integrase, which is responsible for the integration of the expression cassette into the host cell genome [48,50,51]. Thus, *A. tumefaciens* cells, bearing the viral vectors, assume the viral function of primary infection, whereas deconstructed viral vectors promote cell-to-cell spreading, signal amplification and transgene expression [52]. Because it is a model plant, the use of *Nicotiana benthamiana* plants as a host is also one of the advantages of this method, since gene transfer techniques for heterologous expression are already well established, besides providing a high biomass yield and capacity, as well as the ease of infiltration of its leaves [26]. Therefore, the combination of deconstructed viral vectors with bacterial cells capable of transporting and transferring the transgene and a well-established plant system for transformation makes the Magnifection™ technique an excellent alternative to the platforms conventionally used for biosynthesis of protein and peptides recombinant of the pharmacological interest, since their application allows an estimated protein yield between 0.5 and 1 g for each kg of fresh biomass, which represents robust amounts for clinical trials with the biomolecules produced [21,47].

Conclusion

Although microbial resistance is a natural adaptive phenomenon, the indiscriminate use of over-the-counter antibiotics has contributed to the increase in the number of multidrug-resistant strains in recent decades. Thousands of people die each year from infections caused by multi-resistant microorganisms, which is one of the biggest threats to public health worldwide. Due to the increasing number of infections caused by multiresistant microorganisms, it is urgent to prospect for new molecules that can counteract infections and, at the same time, have a lower potential to confer resistance. Therefore, AMPs can be used as a therapeutic alternative, either alone or in combination, to combat strains resistant to various antibiotics. However, PAMs are naturally produced at low concentrations. In recent years, plants have been used as hosts capable of producing biologically active recombinant therapeutic proteins, vaccines, and AMPs. This is because plants have a specific set of desirable characteristics for the biosynthesis for some AMPs to present their correct conformation and be biologically active [21]. That is, recombinant biosynthesis of AMPs in plant systems may be a viable alternative to complement the fight against pathogenic microorganisms. Magnifection™ technology has become an excellent alternative for rapid, scalable and massive biosynthesis of therapeutic molecules, revolutionizing the way drugs are produced. This technology has great potential for increasing the efficiency of pathogen-active molecule production, with possible reductions in biosynthesis time and increased supply of medical products, reducing costs and increasing access to the population.

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Conflict of Interest

All authors declare that there is no conflict of interest

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