Plant genetic engineering: basic concepts and strategies for boosting the accumulation of recombinant proteins in crops

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Abstract: Plant genetic engineering represents one of the most striking biotechnological advances in the production of products useful to man. Among the numerous applications of plant biotechnology, the production of recombinant proteins stands out. These polypeptides may have applications in agriculture, industry, the environment and human health. Several molecular strategies have already been developed to increase the levels of recombinant protein biosynthesis in transgenic plants. This review introduces the fundamental concepts of plant genetic engineering and outlines some of the most important basic strategies for increasing final levels of recombinant proteins synthesized by the enzymatic machinery of common crops.

Keywords – Genetic engineering, recombinant proteins, crops

I. INTRODUCTION

Proteins and their functional importance in plants.

Proteins perform many biological functions and due to their functional versatility are found in the most diverse cellular environments. These biomolecules play a fundamental role in many cellular processes, such as sophisticated regulation of anabolic and catabolic reactions and pathways; in helping to maintain the shape and support of cells and subcellular structures; acting as catalysts for chemical reactions; in the mediation of the signal transduction process; as carriers of molecules and ions against concentration gradients; as primary regulators of gene expression at both transcriptional and translational levels, and even as carbon chain suppliers in extreme catabolic processes of harnessing chemical energy.

Given their importance for the correct individual performance of each subcellular sector and their own dynamic functional integration, proteins are distributed in different compartments and organelles of the plant cell.

Advanced research currently focuses on the study of protein biosynthesis systems and the various aspects related to these biomolecules, from the structural, functional, physicochemical, metabolic point of view and that related to the expression and accumulation of polypeptides in different cell organelles.

In this context, the elucidation of the molecular mechanisms of gene expression regulation in their main stages constitutes one of the main challenges of biological research. Molecular processes that drive an informational and functional sequence of DNA nucleotides to be transcribed into an intermediate molecule, RNA - sometimes itself the end product of gene expression - and translated into a protein correctly transported to its preprogrammed destination, are complex and dynamic, which makes understanding the global process a challenging challenge for science.

As molecular units that encapsulate the genetic information expressed in RNA and proteins, genes were the target of new biological approaches and approaches in the early 1980s, after the discovery that certain organisms, notably bacteria, naturally can transfer genes. for plants, changing the phenotype of the latter. Understanding the main molecular mechanisms governing plant genetic transformation has set precedents for in vitro gene transfer between different organisms, commonly called transgenics.

Genetic transformation is the incorporation of exogenous and inheritable DNA by living organisms through in vitro methods.

Transgenes are functional DNA fragments carrying genetic sequences originating from different organisms (and may even belong to species that do not have sexual compatibility), usually obtained through recombinant DNA technology and introduced naturally or in the laboratory into the genome of recipient species.

Thus, transgenic organisms, such as bacteria, yeast cells, filamentous fungi, plants and animals, are those that contain one or more genes inserted into their genomes by means other than the sexual pathway.
The cells of these organisms can coordinate transcription of transgenes as well as the translation of mature mRNAs into correctly folded proteins, assembled and modified after translation. In this case, the newly produced proteins - called recombinant proteins - are capable of desirably altering the phenotype of gene receptor organisms to achieve a range of practical objectives.
Recombinant protein is a transgene-encoded polypeptide normally synthesized in organisms other than those of its origin. Some recombinant proteins may be encoded by additional copies of transgenes from the host plant itself, introduced into its genome by in vitro techniques.
When the development of transgenic plants, as well as other genetically modified organisms, meets objectives linked to food production or improved procurement of drugs, for example, we can say that they are essentially biotechnological products.
Biotechnology can be defined as the various forms of technology that exploit biological sources - and/or their parts and components to obtain useful products for mankind. Commonly the term is also used to define forms of technology that depend on the use of molecular biology / genetic engineering techniques for the construction of new organisms and / or products for industrial, medical and related applications.
The molecular strategies that advocate recombinant protein biosynthesis, mediated by the enzymatic machinery of higher plants, are the applied examples that hit the market and those most often employed by research groups involving genetic transformation of plants.
A significant portion of research involving transgenic plants is restricted to fundamental experimental validation studies prior to obtaining a product intended for the market. The herbaceous plant Arabidopsis thaliana (L.) Heynh. a crucifer evolutionarily close to the mustard plant [Brassica integrifolia (H. West) Rupr.], falls into the latter case, since it has received model plant status for the experimentation of plant transgenics in a manner analogous to the position achieved by flies. of genus Drosophila sp. for the study of population genetics.
Whether as a result of the expression of one or more recombinant proteins or the suppression / blocking of protein expression and metabolic intermediates of the plant itself or weed pathogens, the objectives governing the production of transgenic plants intended for the market focus mainly on obtaining food. , medicines and industrial products and reagents.
The ability of higher plants to express genes from other organisms (not necessarily evolutionarily close) and to produce recombinant proteins in satisfactory and economically viable quantities are the main factors that characterize them as efficient systems for producing numerous proteins. with the most varied applications.
Applications of recombinant proteins of plant origin fall into two major groups: endogenous and exogenous.
Endogenous application is one that is restricted to the organism of origin of the recombinant protein.
Exogenous application is one whose purpose concerns organisms other than those of origin of a recombinant protein.
Endogenous application, that is, in the transgenic plant itself, is usually related to agronomic aspects and recurrent to human food, such as obtaining resistance to forms of biotic stress, such as the attack of insects and disease-causing pathogens that affect crops. and the storage of agricultural products; tolerance to different forms of abiotic stress - mainly the toxic action of herbicides - and the so-called "functional" foods, which have higher yields of certain oils, vitamins, amino acids and carbohydrates and have higher nutritional value and desirable characteristics. to the diet of human and food rations, such as the decrease of antinutritional / allergenic factors and undesirable for the food industry.
Transgenic plants also function as vehicles to produce proteins and other biomolecules to be extracted from plant material and purified for exogenous application, ie, outside the plant in which they were biosynthesized.
The destinations for use of these true protein biofactories are numerous, ranging from the pharmaceutical industry to that of cleaning and hygiene materials, including the development of recombinant enzymes for strictly industrial application and to the obtaining of new materials (biopolymers) with desirable characteristics. the apparel, surgical and potential employment sectors in the security, naval, air and military sectors.
Transgenic plant breeding is based on three broad sets of cellular and molecular set of techniques: genetic engineering, methods for gene transfer between different organisms, and in vitro culture of plant tissues. This paper focus on genetic engineering.

II GENETIC ENGINEERING

Genetic engineering is a comprehensive set of molecular concepts, techniques, and applications that began in the first half of the 1970s and was developed by the progressive correlation of information important for understanding

the structure and function of nucleic acids, obtained about thirty years ago. rather, and by the discovery of enzymes capable of modifying the molecular structure of nucleic acids, which allowed their manipulation in vitro.

Genetic engineering is the controlled modification of nucleic acid molecules in the laboratory to generate new combinations of genes or sequences (such as fusion proteins). Genetic engineering allows the association of a given gene or genes under the control of different regulatory systems, as well as introducing specific mutations in a given nucleic acid molecule.

Genetic engineering techniques allow the assembly in the laboratory of hybrid DNA molecules containing functional segments of deoxyribonucleic acids from more than one organism, organized in an organized manner, as in a "molecular mosaic" where multiple DNA strands are assembled from one another. in a logical manner that allows each segment to perform its function correctly.

The tools used to manipulate the DNA of different organisms are enzymes capable of structurally modifying these molecules and integrating recombinant fragments (containing different combinations of DNA - hence the name recombinant DNA technology) into circular bacterial DNA molecules called plasmids.

Plasmid is a bacterial extrachromosomal genetic element, often transmissible from cell to cell, which can be propagated both in the cytoplasm and as an integral part of the bacterial chromosome. These circular DNA molecules are not essential for the survival of the bacteria, but contain genes commonly associated with important phenotypic traits, such as antibiotic resistance. Its application is recurrent in genetic engineering in cloning and expression of transgenes.

The DNA modifying enzymes catalyze processes of structural change in the molecule, such as restriction and cleavage at specific sequential sites of nucleotide nitrogen bases and ligation of compatible DNA ends.

The first of these enzymes was purified from cells of the bacterium Escherichia coli (T. Escherich) and characterized in 1967 by Martin Gellert of the National Institutes of Health (NIH) and called DNA ligase. Gellert found that this enzyme was able to catalyze the establishment of phosphodiester bonds between distinct Lambda phage (λ) DNA fragments by linking distinct portions of the virus's DNA molecule.

Just a year later, biochemists Stuart Linn and Werner Arber of the University of Geneva (Switzerland) discovered the catalytic action of DNA restriction enzymes, culminating in the purification and characterization of the first site-specific restriction endonuclease - endonuclease R, later called Hind II.

The use of both enzyme classes allows the specific fragmentation of DNA molecules carrying endonuclease-recognized restriction sites, as well as the binding of the ends of different molecules by reconstituting the covalent fodphodiester bonds between phosphate clusters and deoxyribose molecules. of both ends, mechanism effected by the catalytic action of the DNA ligase enzyme.

The same procedure can be employed for inserting transgenic-containing fragments - including those that confer antibiotic resistance - into the bacterial plasmids previously previously isolated in the laboratory. Transgenic-containing plasmids are then reintroduced into bacteria that accept them as elements of their own genome.

Since transgenic bacterial cells (carriers of the modified plasmid) can replicate under appropriate conditions in a selective culture medium containing the antibiotic against which they are resistant, transgenic carrying plasmids also undergo a replication process, resulting in obtaining new copies of plasmid DNA.

Each replicated bacterial clone contains multiple copies of the plasmid and analogously of the transgene itself. This mechanism was called gene cloning. Once multiplied on a large scale, the various copies of the transgene-containing plasmids are separated from the bacterial cells and purified in vitro to be free of contaminant molecules for subsequent introduction into the nucleus or lumen of plant organelles containing transgenes. their own genomes, such as mitochondria and chloroplasts.

Genetically engineered bacterial plasmids used in plant genetic transformation experiments are also called gene expression plasmid vectors, as they contain recombinant protein coding sequences duly flanked by distinct regulatory sequences as promoters; signal peptides and terminators which, once arranged sequentially and at the same reading stage, constitute the expression cassette to be stably integrated into the plant genome.

The expression cassette consists of one or more genes and their flanking regulatory sequences. Generally, its constituents are the gene promoter, an open reading frame (the coding sequence), and the DNA sequence corresponding to the untranslated mRNA region 3, which contains the polyadenylation site in eukaryotes.

A gene promoter is a segment of DNA that is directly involved in initiating gene transcription, located upstream on the same strand and usually near the coding sequence that it regulates. Present on the promoter are the site of initiation of RNA synthesis and the sites and responsive binding elements of DNA-dependent RNA polymerase and transcription factors, as well as regulatory elements such as transcription activators and repressors.

The signal peptide sequence is a short sequence encoding a short peptide chain, directly involved with the direction and post-translational transport of a protein to organelles or to the secretory pathway of proteins in eukaryotic cells.

They may be associated with either the N or C-terminus of the target protein. Some signal peptides undergo specific enzyme recognition and cleavage after protein transport.
The coding sequence for a gene is the portion of nucleic acid that is transcribed into RNA, which may or may not be translated into a protein.
The transcription terminator is the region that marks the gene termination and contains the transcription stop signal.
DNA cloning techniques were first devised and performed in 1972 by Paul Berg at Stanford University, California, and resulted in the obtaining of a hybrid DNA molecule containing a DNA sequence fragment from a Lambda phage (λ) inserted into the genome of the SV40 tumor-causing virus, which could be inserted into mammalian cells.
Just one year after Berg's achievement, Stanley Cohen at the same university was able to develop a method of inserting exogenous bacterial plasmids into recipient E. coli cells and, along with Herbert Boyer from the University of California, reported the first in vitro construct. of a bacterial plasmid containing genes from two other plasmids previously digested with the restriction enzyme EcoRI.
Once inserted into E. coli cells, the Boyer and Cohen hybrid plasmid was efficient in giving transgenic bacteria simultaneous tolerance to tetracycline and kanamycin antibiotics.

III. CHOICE OF PROMOTERS AND TERMINATORS.

Among the most important plasmid genetic elements related to high levels of transgene expression to be introduced into the plant genome are the promoter and terminator of gene transcription.
Efficient eukaryotic promoters are those capable of inducing high levels of mRNA production because their sequence carries specific sites free of steric hindrances, allowing for the easy coupling of transcription factors and RNA polymerase complex, in addition to the abundant presence of mRNAs. activating elements and facilitated modulation of repressive elements.
As for the type of regulation of transcriptional gene expression, promoters are basically divided into two types: constitutive and tissue-specific (exemplified below).
Constitutive promoters allow the continuous transcription of genes associated with them throughout the organism's life cycle, promoting generalized gene expression in all organs of multicellular organisms such as roots, stem, leaves, flowers, fruits and seeds in plants.
The most widely used constitutive promoters for recombinant expression in dicotyledons are CaMV 19S and 35S, derived from the cauliflower mosaic virus (CaMV) 19S and 35S transcripts, a virus that attacks cruciferous plants such as cauliflower (Brassica oleracea L.) and broccoli (Brassica oleracea L. var. italica Plenck).
In monocotyledons the maize ubiquitin 1 gene promoter (Zea mays L.) (ubi-1) has been used for the expression of recombinant proteins in cereals.
The most widely used terminators for the control of transcriptional gene expression in transgenic plants include 35S CaMV, the nos and ssu genes respectively of Agrobacterium tumefaciens and pea (Pisum sativum L.).
Another class of promoters that emerges as an efficient alternative of efficient transcriptional regulation, especially in plants of agronomic interest, is that composed by inducible promoters susceptible to strictly external regulation, mediated by chemical and physical stimuli.
These regulatory sequences emerge as interesting tools for the sophistication of gene expression at the transcriptional level, as they temporarily restrict it to circumstances of the environment in which the transgenic plant in question is located.
An interesting example is the peroxidase-activated sweet potato [Ipomoea potatoes (L.) Lam.] promoter, which is used for the 30-fold increased production of the recombinant enzyme beta-glucuronidase in transgenic tobacco plants (Nicotiana tabacum L.). the presence of hydrogen peroxide after harvesting.
Another application of this promoter class was observed in the rapid induction of recombinant protein biosynthesis in tobacco (N. tabacum) obtained by the use of the tomato hydroxy-3-methylglutaryl Coa reductase (Lycopersicon esculentum Mill.) (HMGR2) promoter, activated by mechanical stress induced by harvesting practices, a system developed by the now defunct American company Crop Tech Corp.
Tissue-specific promoters: expression of recombinant proteins in seeds.
Tissue-specific promoters are regulatory sequences that restrict gene expression spatially to only one or more parts of the plant and may also indirectly regulate expression at a temporal level when the organ destined for the accumulation of recombinant proteins is associated with only one period of the plant cycle. culture (eg flowers and seeds).
Several tissue-specific promoters have been sequenced and extensively characterized at the molecular level, such as those that control the expression of a corn seed zein (Z. mays), wheat glutenin (Triticum aestivum L.), and rice glutelin (Oryza sativa L.) and pea seed proteins (P. sativum).

Expression of recombinant proteins specifically in seeds entails several advantages naturally provided by such organs.
As processing and purification costs are inversely proportional to the concentration of the product relative to plant biomass, the accumulation of high levels of recombinant proteins in a small volume leads to a significant decrease in the production costs of recombinant proteins as biopharmaceuticals.
Restricting accumulation of recombinant proteins in seeds reduces risks of protein exposure to both herbivory and pollinating insects and rhizosphere microorganisms and reduces the possibility of potential toxicity of the molecules to the plant.
However, the success of this strategy requires flowering and fertilization during the reproductive cycle of the plant to obtain seeds, while proteins produced in vegetative organs can be harvested prior to pollen production, eliminating the possibility of gene flow via flowers.
The cultivation of transgenic plants in a greenhouse and the use of autogamous plants (which suffer predominantly self-pollination) such as soybean [Glycine max (L.) Merr.], Rice (O. sativa) and beans (Phaseolus vulgaris L.), may help to decrease the likelihood of gene flow even when tissue-specific promoters are employed for the expression of recombinant proteins in seeds.
During the collection and extraction of recombinant leaf-expressed proteins, the high natural hydrolytic activity and the presence of proteases associated with these tissues can provide a drastic reduction in the accumulation levels of these molecules.
Unlike leaves, seeds are natural storage sites for high concentrations of reserve proteins used in embryo nutrition in the early stages of seedling physiological development. These organs present not only an appropriate biochemical environment, devoid of phenolic compounds and low hydrolases, but also specialized tissues for highly stable protein accumulation for long periods of time even at room temperature, which reduces the need for special conditions. storage.
An example of this ability is that antibodies expressed in cereal seeds remained stable for at least three years after storage at room temperature, with no detectable loss of activity.
Another application can be verified by the application of a tissue-specific promoter modulating the expression of a bean seed arcelin (P. vulgaris), capable of providing a 36-fold higher accumulation of a monoclonal antibody in A. thaliana seeds when compared to constitutive promoter 35S from CaMV (1% TSP).
Thus, tissue-specific promoters of reserve protein coding genes are an excellent tool to be employed in recombinant protein expression systems in plants.

IV. PROTEIN TARGETING

Transcription and translation levels are directly related to the efficiency of recombinant protein biosynthesis. Another variable should be considered when estimating the production yield or accumulation of recombinant proteins: their level of degradation after biosynthesis, ie, their degree of post-translational stability.
The molecular strategies that act on protein stability, coupled with the choice of promoters, correspond to the most efficient advances in terms of real yield increases of recombinant protein production in transgenic plants and include two distinct approaches taken simultaneously: the use of promoters. tissue-specific - mainly those associated with endogenous seed and tuber genes - and the subcellular addressing of proteins through the above-mentioned signal peptides.
N or C-terminal signal peptides may be fused to recombinant proteins to direct them to specific sites in the cell. These sequences may target proteins to the mitochondria, vacuoles, chloroplasts or retain them in the endoplasmic reticulum and are usually cleaved upon arrival of the protein of interest to the target organelle.
Subcellular addressing plays a key role in the accumulation levels of recombinant proteins, as the cell compartment in which they are found directly influences folding, assembly and post-translational modification processes, and prevents immediate degradation and interference. of proteins with cell metabolism - quite frequent events in cytosol.
The addition of signal peptides for efficient organelle accumulation provided higher levels of recombinant protein production in many studies where they were naturally poorly expressed due to their toxicity.
Commonly four subcellular destinations are the main compartmentalization targets for the production of biopharmaceuticals: the apoplast, the endoplasmic reticulum, the chloroplasts and the seed protein bodies.
We will highlight special attention to this last case, since in this same edition there is a chapter that details the subcellular addressing to the main organelles of plant cells.

Addressing recombinant proteins to protein bodies.

Protein bodies are extensions derived from the endoplasmic reticulum that specialize in the accumulation of reserve proteins. They function as compartments that occupy a large cytoplasmic volume and evolutionarily have undergone adaptations resulting in the ability to compartmentalize large volumes of proteins and maximize the integrity of these proteins.

These globular inclusions are found in cotyledonary cells of tobacco seeds (N. tabacum), legumes such as soybean (G. max) and beans (P. vulgaris), as well as grasses such as wheat (T. aestivum) and barley (Hordeum vulgare L.), and originate when the reserve protein biosynthesis begins in the tissues responsible for nourishing the embryo.

Thus, there is a significant increase in the number and availability of protein bodies throughout the physiological maturation process of the grains of these species.

Since these highly specialized vacuoles do not fuse with lysosomes, their lumen has a near neutral pH and is practically absent from aminopeptidases, factors that characterize them as a subcellular environment where protein degradation is minimal and an excellent target for depletion. addressing recombinant proteins when the main goal of genetic transformation is to maintain the stability of these molecules.

Protein bodies of soybean seeds (G. max) contain approximately 70% of the grain proteins, and most of this percentage refers to the glycine reserve protein, the most abundant among the proteins present in these seeds.

Some molecules have already been stably successfully accumulated in soybean protein bodies (G. max), including the enzyme D-myo-inositol-3-phosphate synthase (MIPS), which is involved in phytic acid biosynthesis in seeds; a corn δ-zein reserve protein (Z. mays) and the E. coli thermolabile toxin B (LT-B) subunit.

Several studies aiming at the expression of biopharmaceuticals, recombinant antibodies and microbicides in plant systems, notably seeds of transgenic soybean (G. max) plants, have been developed by the group of EMBRAPA's Synthetic Biology Laboratory (SBL).

Molecules such as human growth hormone, breast cancer marker antibody fragments and a potent anti-HIV microbicide have already been successfully accumulated in soybean (G. max) seeds using an expression strategy that combines the tissue-specific promoter of β-Faseolin seeds of bean (P. vulgaris) fused to the α-Coixin signal peptide of the Coix lacrima-jobi L. plant, a nascent protein addressor for soybean (G. max) cotyledon protein bodies.

V. CONCLUSIONS

Demand for proteins of agricultural, veterinary, industrial and therapeutic interest has increased in recent years. Genetic engineering has been able to develop, in the last two decades, transgenic plants capable of presenting useful phenotypes for humans, with great impact on the improvement of material living conditions.

The limitations of transgene expression are a major challenge for breeders and genetic engineers. Strategies for increasing gene expression levels in transcription and translation are becoming increasingly interesting to circumvent these obstacles.

With the first products of modern plant biotechnology reaching the final stages of pre-commercial development and some on the market, the strength of plant biotechnology is present and opens new fronts and possibilities for obtaining more and better useful molecules for society.

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