Production of Foreign Compounds in Transgenic Plants

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I. INTRODUCTION

Expression of bacterial genes, mammalian genes, or other plant genes is becoming routine for several plant species. Most transgenic plants are generated for basic research purposes, such as the evaluation of tissue-specific expression of genes, processing and secretion signals, and enhancer elements. A growing effort is being made to use transgenic plants for broader objectives. One of the most successful approaches has been to engineer resistance against insects, viruses, and other pathogens, as well as herbicides. Numerous examples of resistance mediated by expression of bacterial or viral genes are available (see Chap. 11–13).

Crop improvement by engineering for specific traits can also be achieved through production of enzymes and metabolites encoded by foreign genes. Thus, the amino acid composition of seed proteins has been altered and, for example, transgenic canola, expressing a Brazil nut albumin, contains up to 33% more methionine in its seed protein [1]. The alkaloid composition of some medicinal plants has been improved. The production of scopolamine, a medicinally important anticholinergic drug is highly increased in transgenic Atropa belladonna plants expressing hyoscyamine 6β-hydroxylase from Hyoscyamus niger, which catalyzes the conversion of the precursor hyoscyamine to scopolamine [2]. The shelf life of fruits and vegetables can be extended. Tomato expressing a bacterial ACC deaminase gene reduces ethylene synthesis, and the mature fruits remain firm for at least 6 weeks longer than the nontransgenic controls [3]. Flavor can also be enhanced, transgenic lettuce and tomato have been obtained that express monellin, a sweet protein isolated from some African berries that elicits a flavor approximately 100,000 times sweeter than sugar on a molar basis [4]. The freezing properties of harvested produce can be improved and, in tomato plants expressing an antifreeze protein from the polar fish, winter flounder, ice recrystallization is inhibited [5], making accumulation of the protein cold-specific [6]. The toxicity of some vegetables can be reduced. More than 70% of human cadmium intake is contributed by vegetable products. Shoots of transgenic tobacco expressing the mouse metallothioneine I gene contain about 24% lower cadmium
ally, whole organisms or crude preparations are used, when relatively low purities are required. Transgenic plants may constitute an appropriate system to produce industrial enzymes, because the purification costs may be lowered owing to the absence of bacterial contaminants. A couple of examples of transgenic plant-produced enzymes are available.

Hoekema’s group have expressed *Bacillus licheniformis* α-amylase in transgenic tobacco [31]. The enzyme α-amylase hydrolyzes α-1,4-glycosidic linkages in the starch components amylose and amylopectin [32], and it is used in the starch-processing industry, and also in others, such as the brewing, baking, wine and juice, and detergent industries. *Bacillus licheniformis* α-amylase is the most commonly used in starch liquefaction because of its heat stability and wide pH range of activity [33]. To express the enzyme in tobacco they used constructs containing the α-amylase sequence preceded by an enhanced CaMV 35S promoter and either the enzyme’s signal peptide or the sequence encoding the signal peptide of the tobacco PR-S protein. Both signals functioned equally well in secretion of the α-amylase to the extracellular space and were cleaved correctly on secretion. The molecular mass of the enzyme produced in tobacco was 64 kDa; this value is higher than that of the *B. licheniformis* enzyme, which is only 55.2 kDa. This difference could be attributed to glycosylation in plants, as the protein’s primary structure presents six potential asparagine-linked glycosylation sites [33]. Deglycosylation experiments with *endo-N*-acetylgalactosaminidase H and trifluoromethanesulfonic acid showed that the incorporated carbohydrates were of the complex type. Complete deglycosylation reduced the molecular mass to 55 kDa.

Despite differences in glycosylation, the enzyme exhibited biological activity and had similar thermostability. It accumulated up to 0.3% of total soluble protein, with an expression level of 0.2% in seeds. To test the correct characteristics of the enzyme, it was applied in starch liquefaction. Milled transgenic tobacco seeds were added to corn and potato starches. HPLC analysis of the hydrolysis products showed no differences between the bacterial and the plant-produced α-amylases. The enzyme did not need any further purification.

Transgenic plants expressing α-amylase were phenotypically indistinguishable from nontransgenic tobacco plants, and leaves contained similar amounts of starch. This suggests that the α-amylase was located extracellularly and, consequently, it had no access to the intracellular starch. The production of transgenic potatoes expressing α-amylase is an interesting possibility. No degradation of the starch should occur until homogenization of the tubers, as enzyme and substrate are in separate locations. The α-amylase levels obtained in tobacco would be sufficient to hydrolyze the starch present in the tuber, and no addition of exogenous α-amylase should be needed.

Also Hoekema’s laboratory has expressed *Aspergillus niger* phytase in transgenic tobacco [34]. Phytase catalyzes the conversion of phytate (*myo*-inositolhexaphosphate) into inorganic phosphate and *myo*-inositol [35]. Phytate is the main storage form of phosphorus in many plant seeds used as animal feed, but it is a poor nutrient for monogastric animals and, consequently, inorganic phosphate has to be added to the animal diet. The addition of phytase to feed constitutes an alternative; it optimizes phosphorus utilization and reduces excretion of phosphate by poultry and pigs [36,37]. A phytase from *A. niger* has been developed that functions well in the acidic conditions found in the gastrointestinal tract of these animals.

Tobacco was transformed with constructs containing the *A. niger* phytase-coding sequence, preceded by an enhanced CaMV 35S promoter and the sequence encoding the signal peptide of the tobacco PR-S protein. Seeds of transgenic plants were assayed for phytase activity and showed accumulation of the enzyme to 1% of soluble protein. The plant-produced enzyme had a molecular mass of 67 kDa, which is smaller than that of the *aspergillus* enzyme. The difference was due to different glycosylation, as the protein presents a total of ten potential
asparagine-linked sites, and the aspergillus enzyme is heavily glycosylated [38]. Deglycosylation of the protein resulted in a molecular mass of 60 kDa for enzyme from both sources.

Transgenic phytase seeds were tested in vivo by milling and subsequent addition to the basal diet of broilers. Diets supplemented with nontransgenic seeds, diets with and without added inorganic phosphate, and diets with added commercial phytase were used as controls. Diets supplemented with transgenic seeds resulted in significantly higher growth rates than those with control seeds or no addition, and were comparable with those with added inorganic phosphate or added phytase.

The plants expressing phytase were indistinguishable from controls, and germination of the seeds was not affected by the presence of the enzyme. The high expression levels indicated stable accumulation in seeds, and activity was not affected after 1 year of storage at room temperature of the milled seeds. Tobacco seeds can be applied in animal diets, but the enzyme could well be expressed in seeds from crops used in animal feed, thereby improving their nutritional value.

V. VACCINES

The expense of immunization programs in developing countries becomes prohibitive when large populations need to be treated. Plants can represent a less expensive production system here, being most appropriate for oral vaccines that could be expressed in edible plant tissues. Hepatitis B surface antigen (HBsAg), which is used as a vaccine against hepatitis B, has been successfully expressed in transgenic tobacco plants [39].

Hepatitis B virus infection is a widespread viral infection of humans that causes hepatitis and hepatocellular carcinoma [40]. The serum of infected individuals contains 22-nm, noninfectious viral particles, composed of elements of the viral envelope, including the HBsAg [41]. HBsAg for use in vaccines was purified from the serum of infected individuals until a recombinant form was expressed in yeast [42]. Intramuscular injection of HBsAg results in effective immunization and protection from viral infection in healthy individuals [43].

Tobacco was transformed with constructs containing the sequence encoding for the HBsAg preceded by an enhanced CaMV 35S promoter and the 5'-untranslated leader of tobacco etch virus [44]. The presence in transgenic leaf extracts of material that reacts specifically with monoclonal antibody against HbsAg was tested, and levels ranging up to 66 ng/mg of soluble protein were found. HBsAg was purified by immunoaffinity chromatography; negative staining and transmission electron microscopy revealed the presence of particles the average diameter of which was 22 nm, similar to the particles observed in human serum. This indicated that the plant-produced HBsAg is properly processed and retains the capacity for self-association in the highly immunogenic particle form.

The maximal levels of HBsAg obtained were approximately 0.01% of the soluble leaf protein, which is insufficient for the efficient use of plants as a production system for HBsAg. However, if these levels could be increased, the inexpensive plant-produced protein could be used in vaccination against hepatitis B.

VI. INDUSTRIAL PRODUCTS

Plants can also be engineered to produce foreign metabolites by introducing genes coding for the appropriate enzymes that convert compounds that are normally present in the plant into products of higher commercial value.
Cyclodextrins are cyclic oligosaccharides of six (α), seven (β) or eight (γ) α-1,4-linked glucopyranose molecules [45]. They have an apolar cavity and are capable of forming inclusion complexes with hydrophobic substances, thus providing new properties to the complexed molecule, such as improved stability or higher water solubility. They are used in the pharmaceutical industry as delivery systems and in the food industry for flavor and odor enhancement and for removal of undesired compounds, such as caffeine [46]. Cyclodextrins are prepared in vitro by the action of the bacterial enzyme cyclodextrin glycosyltransferase (CGT) on prehydrolyzed starch [47]. The gene encoding cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* has been cloned [48] and has been expressed in transgenic potato [49]. A tuberspecific promoter from a class I patatin gene [23] was used to restrict expression of the enzyme to the tuber, which stores large amounts of starch, the CGTs substrate. The protein was targeted to the amyloplast by using the transit peptide of the small subunit of ribulose bisphosphate carboxylase, which directs targeting to the chloroplast [50], an organelle that is developmentally related to the amyloplast.

Northern blot analysis of the transformants showed transcription of the chimeric gene, but no CGT was detected in tubers by immunoblot analysis. Tubers were analyzed for the presence of cyclodextrins, which can be purified by affinity chromatography and separated by thin-layer chromatography. Expression levels of 2–20 μg α-cyclodextrin 2–5 μg β-cyclodextrin per gram of fresh weight were obtained. The approximate ratio of α/β cyclodextrin is similar to that obtained in vitro with *K. pneumoniae* enzyme [51]. The presence of cyclodextrins indicated that the enzyme was correctly targeted to the amyloplast, for starch synthesis occurs only in this plastid [52].

A potato tuber contains approximately 14% starch, of which 0.001–0.01% was converted to cyclodextrins. For the commercial production of cyclodextrins, this value should be increased to 1–10%. This might be possible by using stronger promoters and more efficient targeting signals.

Plastics are probably the most used materials in industry nowadays. Their use creating environmental problems, because most of them need extremely long periods to biodegrade. Therefore, growing efforts are being made to obtain inexpensive biodegradable plastics.

Poly-D(-)-3-hydroxybutyrate (PHB) is a high molecular weight aliphatic polyester that is accumulated by many species of bacteria as storage material [53] and is a biodegradable thermoplastic. However, the cost of PHB produced by bacterial fermentation is rather high. In the bacterium *Alcaligenes eutrophus*, PHB is derived from acetyl-coenzyme (Co)A by a sequence of three enzymatic reactions [54], catalyzed by three different enzymes, 3-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB), and PHB synthase (phbC). The genes encoding the three enzymes have been cloned, and expression in *Escherichia coli* leads to PHB production. 3-Ketothiolase is also found in the cytoplasm of higher plants, involved in the synthesis of mevalonate, and genes encoding phbB and phbC have been introduced into *Arabidopsis thaliana* [55]. Plants were transformed with constructs containing the coding sequences of either phbB or phbC, preceded by the CaMV 35S promoter. Leaf extracts from transgenic plants that had the phbB exhibited acetoacetyl-CoA reductase activity, whereas transgenic plants containing phbC had no detectable PHB synthase activity, although proper integration and transcription of the gene were observed. The same lack of activity was observed when the enzyme was expressed alone in *E. coli* [56].

Homozygous transgenic plants expressing phbB and phbC were cross-pollinated. The presence of PHB was observed in the hybrid plants by gas chromatography and mass spectroscopy analyses. The highest amount of PHB accumulated in leaves was approximately 100 μg/g of fresh weight, and it formed electron-lucent granules, the size and appearance of which were
similar to the granules found in bacteria. PHB was expected to appear in the cytoplasm, but granules were also observed in the nucleus and the vacuole.

Plants expressing phbB and phbC genes were smaller and produced fewer seeds than nontransgenic plants. This phenotype could be the result of the diversion of acetyl-CoA or acetoacetyl-CoA away from their essential biochemical pathways. Regulation of tissue specificity, time of expression, and cellular localization of the enzymes might avoid these phenotypic effects.

VII. ANTIBODIES

Of the variety of bioactive compounds expressed in transgenic plants, antibodies may offer the widest range of possible applications. An antibody, taken out of the context of an animal immune system, is simply a complex protein capable of binding, with high affinity, a single antigen. That antigen can be anything from another protein to a synthetic organic molecule. Applications that have been envisioned in the plant and that depend solely on the binding affinity of the antibody include pathogen resistance (viral, fungal, and insect), and modulation of metabolic pathways to produce new developmental or nutritional characteristics [57]. Production of antibodies by plant cells offers a variety of new possibilities for basic research in plant biology as well as for large-scale production of antibodies for use as therapeutic, diagnostic, or affinity reagents. The unparalleled capacity and flexibility of agricultural production suggests that antibodies derived from plants may be significantly less expensive than antibodies from any other source. Moreover, antibodies in plants may become useful reagents for isolating and processing environmental contaminants or industrial by-products.

Antibodies produced in plants possess all of the characteristics and are virtually indistinguishable from antibodies produced by hybridoma cells. Recently, it has been found that antibodies that possess catalytic capabilities can be isolated from mouse hybridoma cells [58]. This opens the possibility of introducing new catalytic capabilities into plants or for production of catalytic antibodies useful in industrial processes.

The high capacity and flexibility of agricultural production offers a number of advantages for obtaining antibodies. Genetically stable seed stocks of antibody-producing plants can be isolated and stored indefinitely at low cost; the seed stock can be converted into a harvest of any quantity of antibody within one growing season. Although tobacco has been used as the principal research tool to initiate the study of antibodies in plants, there may be more appropriate plants for production. A variety of common crop plants can be used as the production host. Acreages of perennial forage crops could be generated by clonal propagation or from seed and harvested numerous times in a growing season. The choice of species may depend on the quantity and nature of contaminants encountered during purification. Some candidates are alfalfa, soybean, tomato, and potato. Because large-scale production of antibodies is not yet commonplace, appropriate techniques for purification of hundreds or thousands of grams have yet to be perfected.

As in other eukaryotic cells, secretion of proteins from plant cells is accomplished by an endomembrane system. Recognition and initial processing of proteins destined for secretion occurs in the endoplasmic reticulum and subsequent processing and vesicularization is a function of the Golgi apparatus [59]. We know little about the fidelity with which secreted proteins from evolutionarily distant organisms can be recognized and processed by a plant host endomembrane system. Although signal sequences for secretion generally possess common features, the amino acid sequences are not highly conserved [60]. Data from heterologous in vitro translation of mRNAs have suggested that although there are similarities between animal
and plant translocating systems, they are not truly homologous [61–63]. In addition, once association of a heterologous protein with endomembrane has occurred, there are a variety of processing alternatives that could yield a protein different from the one synthesized in its native host [64]. For example, for immunoglobulins, an unusual glycosylation pattern of an antibody produced in yeast resulted in a structure that did not bind complement [65].

Subsequent to endomembrane association, proteins in plants can be subject to proteolytic processing, assembly with other subunits, glycosylation [66], as well as other possible modifications [64]. Little is known about the ability of plant cells to perform these functions on gene products derived from other organisms because relatively few transgenic plants have been regenerated that express, in abundance, proteins from animals or microorganisms. For that reason, we have chosen to evaluate some of the characteristics of murine antibodies produced by plant cells that could result from association with the plant endomembrane system.

Production of the functional antibody was completely dependent on the presence of the mouse signal sequence at the NH₂-terminus of the immunoglobulin. In the absence of the signal, immunoglobulin accumulation was very poor and heavy chain–light chain complexes were not detected. Dramatic increases in immunoglobulin accumulation were observed when signal sequences were included as well as when the individual chains were coexpressed in the same plant. These results suggested that the mouse signal sequences affected endomembrane localization, assembly, and possibly secretion of the antibody.

To optimize the secretion of immunoglobulins from plant cells, we designed an expression strategy to exploit secretion pathways known for other eukaryotes. We have replaced the native mouse leader sequence with a yeast signal peptide and prosequence from the α-mating factor peptide oligomer. In other eukaryotes, many peptide hormones are derived from precursors containing long prosequences, terminating in Lys-Arg residues that are removed before secretion [67]. A specific protease resident in the endoplasmic reticulum is responsible for cleavage [68]. Our results suggest that a similar protease, which selectively recognizes prosequences terminating in Lys-Arg pairs, is present as an integral membrane protein of tobacco leaf cells.

VIII. CONCLUDING REMARKS

A wide variety of foreign products can be obtained from transgenic plants, not only proteins, but also all kinds of modified compounds through the expression of the appropriate enzymes or even catalytic antibodies. The production of plant biomass is inexpensive, the plant cell machinery can provide specific processing unavailable from microbial systems, the isolated products are free of bacterial contaminants, the plant seeds can be used as a low-cost storage, and ultimately plants use sunlight as their source of energy. These facts make us believe that the production of foreign compounds in plants is becoming a competitive alternative system to traditional methods.

REFERENCES


