



September 2010

RISK MANAGEMENT

Modifying Plant Growth
the Cisgenic Way

.....1

The Safety Assessment
of Transgenic Plants in
which Gene Expression
Has Been Modified

.....5

Large-scale
Molecular Farming of
Recombinant Human
Collagen in Transgenic
Tobacco

.....8

Modifying Plant Growth the Cisgenic Way

Venkatesh Viswanath and Steven H. Strauss

As genomics has progressed to include a much wider variety of organisms than the few model species that were widely studied in the past, the ability to use native genomic information for transgenic modification has become widely available. In a report in *Plant Biotechnology Journal* on the first use of cisgenes intended to modify the growth of plants, the authors found that the transfer of entire native genes that play roles in biosynthesis or signaling of gibberellic acids (GAs), including their 5' and 3' proximal regulatory regions, impart changes in growth rate and stature in poplars¹. This essay summarizes their work and evaluates its possible utility for plant breeding.

What are cisgenes and intragenes?

As transgenic technology matures and diversifies, it is useful to have terminology that reflects its growing diversity. Cisgenes are a subset of intragenes. An intragenic plant, produced by insertion of an intragene, is defined as “a transformed plant that only contains genetic elements derived from within the sexual compatibility group”², but does not constrain their order, arrangement, or preclude small changes in sequence or expression. Thus, introduced point mutations, promoter/coding region swaps, and the use of RNAi, amiRNA, or antisense suppression, are all legitimate. In contrast, cisgenes are flanked by their native regulatory regions, including their introns, and thus the gene is truly a part of a conventional breeder’s gene pool³. In intragenics (but not in cisgenics)³ in which *Agrobacterium* is used, plant-derived T-DNA border sequences (called P-DNA) that closely resemble *Agrobacterium* border sequences are employed so that the claim can be made that all DNAs inserted are of compatible plant origin in sequence. In addition, selectable marker or reporter genes are not included or are removed after transfer by segregation. Recombinases can also be used for marker gene removal, but they do not fully remove all traces of gene presence (the target recombination sequence). However, as they are similar in length to T-DNA borders, it is also likely that P-DNA-like target sequences can be identified if needed. Cisgenes as well as intragenes add to existing genetic diversity due to “position effects” from their insertion; these modify the intensity and pattern of gene expression as a result of their unique chromosomal position and interaction with regulatory elements¹. Thus, in both cases, genetic diversity in expression is increased compared to that of the progenitor genes.

Why the interest?

Although breeders of many types of annual crops can make dramatic changes in genetic composition in a short time period, many other kinds of plant species are very difficult to breed. Thus, making use of native genetic variation, especially where strong domestication

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phenotypes are sought, can be very slow and difficult. This is obviously mostly true for woody plants, which do not flower for a number of years, are intolerant of inbreeding, and are highly heterozygous (masking the expression of desired recessive alleles). But it is also true for many other types of plant species, especially when they are naturally sterile or are part of a highly desired and commercially widespread clone whose genotype needs to remain intact. Examples include potato, apple, grape, and banana⁴. For example, intragenesis has been applied to the development of non-browning versions of established apple varieties (such as Gala, Fuji, Golden Delicious, and Granny Smith) by the silencing the polyphenol oxidase gene. These apples (named Arctic™ apples because of the color of their skin) have been developed by Okanagan Specialty Fruits and tested in the field since 2004. The company has already petitioned the Animal and Plant Health Inspection Service (APHIS) for deregulation of the product in the USA⁵, and plans to do the same in Canada later in the year.

In addition, desired alleles, such as dominant alleles for size reduction, pest tolerance, or specific fruit or nutritional qualities, can be rare or unavailable. Introgression breeding using genetic transformation for disease resistance can benefit from the avoidance of linkage drag⁴, and disease resistance alleles can be more rapidly stacked to provide broader or more durable forms of resistance. Because of the lack of linkage drag, cisgenic plants are likely to be as safe as or safer than those produced with the same genes through traditional or mutation breeding⁴. In forest trees such as American Chestnut that have been devastated by exotic diseases, stacking several resistance alleles obtained from interspecies hybrids via conventional and marker-aided breeding, while also restoring the majority of the American Chestnut genome to promote adaptability, would be a very formidable challenge in the absence of transgenic capabilities.

There are also good social reasons to differentiate cis- and intragenes from conventional transgenes. The concept of transgenic organisms and transgenic food is troublesome for many people, which is reflected by their stringent regulation throughout the world. The public is considerably more comfortable with the idea of a cis/intragenic crop when compared with a transgenic crop⁶. For example, a survey in Mississippi showed that 81% would eat a cis/intragenic vegetable, as compared to only 14 – 23% for a transgenic vegetable [containing genes from non-plant sources]⁷. Similarly, a nationwide survey in the United States found that 52 – 77% would eat a cis/intragenic vegetable (depending on number of genes inserted and source of the gene); whereas only 17 – 25% would eat the same vegetable if it contained a gene from a microbe (bacterium/virus/fungus) or an animal⁸.

Modification of tree growth using cisgenesis

In a proof-of-concept study in which only the growth-modifying “active ingredient” genes, and not the entire T-DNAs, were cisgenic, it was shown that tree growth and architecture could be significantly modified using GA-associated cisgenes¹. The main goal of the study was to examine the feasibility of using cisgenes to modify gibberellic acid (GA) action and hence growth and architecture in poplar tree. Gibberellic acid is a plant hormone with a wide variety of

functions in controlling plant growth and development. Five different cisgenes (*GA20ox7*, *GA2ox2*, *GAI1*, *RGL 1_1*, and *RGL 1_2*) were studied, along with empty vector controls and non-transgenic controls. *GA20ox* is an enzyme that catalyzes the penultimate step in the biosynthetic GA pathway, and thus tends to promote cell division and elongation, whereas the other genes tend to repress or attenuate active GA actions (GA degradation by *GA2ox2*; the other genes were DELLA domain proteins that attenuate GA signals).

Several interesting, statistically significant results were obtained in this study. Plants transformed with *GA20ox7* cisgene had a higher rate and frequency of regeneration of transgenic shoots during antibiotic selection. This suggests that this gene might be useful as a general transformation enhancer. It also dramatically promoted early height and diameter growth on transformants grown in the greenhouse; after six weeks from the date of transplantation, the average stem volume of the *GA20ox7* transformed plants increased by 40% compared with the transgenic (empty vector) controls (**Fig. 1**). *GA20ox7* gene expression was also statistically associated with the growth enhancement (**Fig. 2**). In as yet unpublished work, the researchers also showed that the levels of active GAs increased in the transgenic lines. The growth improvement due to the *GA20ox7* gene, however, diminished over time. The authors concluded that this might have occurred due to the rapid growth and limited pot size in the greenhouse.



Figure 1. Comparison of *GA20ox7* transformed plants (left group) to the empty vector controls (right group) after six weeks from the date of transplantation to begin the greenhouse trial. The transgenic plants had an average stem volume that was increased by 40% compared with the controls.

However, it might have also resulted from a transitory effect of the cisgene, such as from stimulation of cell division but not cell enlargement. The faster growing trees had similar internode lengths to the control trees.

The GA inhibitory genes had variable effects, but generally retarded plant growth. Plants transformed with *RGL 1_2* gene had a reduced rate of regeneration of transgenic shoots and a reduction in growth rate, but had longer stem fiber lengths. *GA2ox2* and *GAI1* transformed plants also had semi-dwarf phenotypes in the greenhouse, while *RGL 1_1* plants appeared similar to wild type. *RGL1_1* transformed plants, however, had reduced leaf size. In their discussion, the authors emphasized that the results were preliminary and require verification in field environments and with a greater diversity of genotypes, as would occur with normal plant breeding.

Potential uses of cisgenics

Clearly, cis/intragenics can be used to modify plants similarly to conventional breeding, but in many cases appear to be able to do it faster and with more specificity. This efficiency will often enable applications that would otherwise be impractical and unaffordable because of the costs and time frames involved (e.g., when very rare recessive mutants must be sought, many alleles stacked, or difficult hybrids generated and backcrossed). For trees, the ability to speed breeding by transfer of genes among related species for resistance to pests could have very high value, given the proliferation of pest

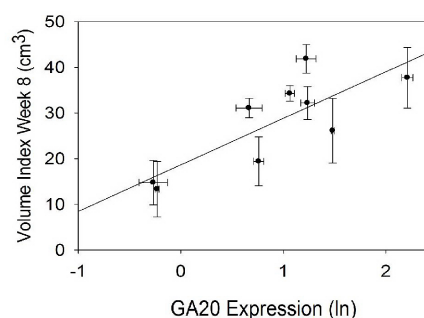


Figure 2. Statistically significant association of growth rate with *GA20-oxidase* gene expression ($p < 0.05$). Bars represent standard errors.

problems in planted and native forests due to climate and other anthropogenic stresses, and the growing proliferation of exotic pest species. Other possible uses include speeding growth rate for bioenergy applications; reducing stature of well-known varieties for ornamental or horticultural applications; improving abiotic stress tolerance by modifying expression of stress tolerance pathway control genes; modification of flowering and induction of sterility; and modification of the quality and nutritional value of ornamental and food products. For example, work is currently underway to produce apples with red flesh⁹. These apples are more pleasing to the eye than the normal apples and contain antioxidants which may provide a direct health benefit to consumers.

Can cis/intragenics avoid the regulatory thicket of transgenics?

Despite many possible uses, the realm of application of cis/intragenics, when compared to transgenics, is highly limited. For example, cisgenics clearly cannot impart new pest tolerance mechanisms, new industrial and pharmaceutical products, or new metabolic pathways to enhance plant nutrition and adaptation. Thus, cis/intragenics should not be viewed as an

alternative to transgenics, but as a tool for extension of traditional breeding when dealing with difficult traits and species. It is also a tool with which the public has more comfort and thus might be used with much more freedom and lower cost than transgenics. A strong case has been made for cisgenic plants to come under a new regulatory tier with reduced regulatory oversight or to be exempted from GM regulation¹⁰. Of the current regulatory systems, to our knowledge, only Australia excludes intragenics from regulation⁶. The Animal and Plant Health Inspection Service (APHIS) in the United States had considered a lower regulatory tier for cisgenic plants in its revised regulations¹⁴, but more recent actions suggest that this proposal is no longer viable. If, instead of improved efficiency, cis/intragenics bring the enormous regulatory, political, and market obstacles of transgenics to what is in essence a modification of conventional breeding, it is unlikely to be pursued for the large majority of potential applications. Unfortunately, some authors have indeed suggested just this⁶. Thus, despite their obvious benefits and high level of familiarity, unless accompanied by regulatory reform, cis/intragenics may be largely avoided rather than embraced.

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The Safety Assessment of Transgenic Plants in which Gene Expression Has Been Modified

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Engineered crops have become a significant component of modern agriculture. Prior to release for commercial planting, a thorough pre-market regulatory review focuses on any potential agricultural and environmental impacts of genetically engineered crops, as well as any differences in food safety that may be associated with the introduction of novel genes and their products. The regulatory review process is a comparative one in which differences between a new transgenic crop variety and its conventional counterparts are assessed, followed by a determination if any changes that have occurred have introduced new risks or heightened existing risks. To date, the great majority of transgenic cultivars that have passed regulatory review contain genes that encode proteins that confer desired novel traits such as insect or herbicide resistance.

Alteration of endogenous gene expression can be an alternative method of producing useful phenotypes in plants. For example, RNA-associated mechanisms can be used to switch off genes, while up-regulation of specific transcription factors can be used to enhance expression and thereby modify a plant's growth or response to stress. Since neither of these two mechanisms necessarily depends on the expression of a new heterologous protein(s), it is reasonable to ask if the safety assessment paradigm developed for and applied to transgenic plants that express novel proteins is appropriate for genetically engineered plants in which gene expression has been altered.

This article briefly summarizes the conclusions of a recent paper¹ that examines the suitability of the currently used comparative safety paradigm to crops in which gene expression has been altered. Parrott et al. (2010) also serves as an up-to-date review of the safety assessment process.

The Current Safety Assessment Paradigm

The development of a new cultivar, whether transgenic or not, involves repeated selection and culling of candidate plants that do not conform to a long list of crop-specific phenotypic traits found in near isogenic

and appropriate comparators, for example: (1) germination and seedling emergence; (2) vegetative vigor; (3) time to anthesis; (4) plant height at maturity; (5) time to maturity; (6) pollen characteristics; and (7) yield. Agronomic and other characteristic traits for each crop have been summarized by OECD, EFSA, and other sources so that cultivars that are advanced into the safety assessment process are the product of repeated selection that contributes to the elimination of both undesirable and unintended variations.

A keystone of safety assessment is an examination of compositional and nutritional equivalence of a transgenic cultivar in comparison to closely related counterparts. A unique set of key nutrients, toxicants, and anti-nutrients associated with each crop are analyzed. Although changes in composition do not necessarily pose new risks, to date the great majority of the crops that have received regulatory approval are compositionally indistinguishable from their conventional counterparts. This has caused some people to question if composition studies are needed for transgenic plants that express simple novel protein-mediated phenotypes, such as insect or herbicide resistance².

A second key focus of contemporary safety assessment is a direct evaluation of the safety of any newly inserted novel proteins with respect to their potential for eliciting toxic or allergic reactions. Bioinformatic comparison to known toxins, anti-nutrients, and allergens, protein digestibility assays, and in vivo studies, such as testing acute toxicity in mice and subchronic toxicity in rats, can be used to assess the potential for adverse effects. Through almost two decades of experience, perceptions about which studies provide significant insights into protein safety, and which do not, have—perhaps not surprisingly—changed. It has been recommended that the assessment could be simplified by a selective and tiered approach⁴; however, the studies required by regulatory agencies have simply become more numerous and more complex with passing time^{3,4}.

Other safety studies include characterization at the

molecular level of both the DNA and any introduced proteins, the safety of novel molecular markers used for selection, and nutritional studies in animals. The authors conclude with respect to the current assessment process:

“This safety assessment paradigm is based on the state of knowledge that existed more than 20 years ago. Some aspects have been found to have a scientific basis, whereas other elements of the safety assessment have provided little relevant information on safety.”

Modulation of gene expression in plants

Parrott et al. (2010) leads the reader to the conclusion that the safety assessment paradigm is itself in need of simplification derives directly from a comparison of conventional breeding with genetic engineering. The authors observe that conventional breeding gives rise to a large number of mutations that typically remain uncharacterized—this is particularly the case where chemical or radiation-induced mutagenesis has been used. Furthermore, a number of studies are cited that demonstrate that transgene insertion can produce less DNA disruption than other conventional breeding methods. While it has been argued that transgenic insertion is *per se* mutagenic, it appears that DNA alteration mediated by naturally occurring transposition and retro-transposition in plants far exceeds that caused by mutagenesis. Parrott et al. (2010) point out that conventional breeding is generally regarded as safe, in spite of the fact that the nature of the changes in new conventional cultivars are usually unknown. The reader is left to wonder why transgenic crops are subjected to such careful regulatory scrutiny.

Small RNA This point is well illustrated by the observation that RNA interference and RNAi species are natural components of the genome that have been historically, albeit unknowingly, exploited by plant breeders. The plant genome potentially encodes

thousands of small regulatory RNAs. Traits as varied as the buff seed coat in soybeans and the stems of some modern maize hybrids depend on small RNA-associated mutations. The FLAVR SAVR tomato, high-oleic soybeans, modified starch potato, low allergenic ryegrass, low nicotine tobacco, and various virus resistant crops are all examples of genetically engineered plants in which gene modulation mediated through small RNAs has produced a desired phenotype in the absence of a novel heterologous protein. Several of these were approved and commercialized prior to elucidation of the small RNA mediated mechanism by which the phenotypic changes had occurred.

RNAi-associated changes can be targeted to virtually any gene, even if present in multiple copies; plant scientists have focused their attention in particular on genes associated with pests and pathogenicity. Other targets include altered growth and development, altered nutritional content, and elimination of undesirable compounds. No exogenous genes are used in this form of genetic engineering, and if fact, no genes need be introduced if selectable markers are not present.

“Many of the most important traits associated with crop domestication and improvement have been mediated by alterations in TF expression;...”

Transcription factors Transcription factors (TF) and other gene-signaling pathway modifiers have not been used to date in commercial cultivars; however, they have great potential to modulate plant development and stress responses. These proteins, which can enhance or repress gene expression, are key regulatory molecules that constitute up to 10% of plant genes. The effects of a single TF need not be restricted to a single gene; expression of some TFs can trigger a cascade of events. Many of the most important traits associated with crop domestication and improvement have been mediated by alterations in TF expression; for example, loss of shattering of grain crops, loss of branching in maize, naked grains in maize, and dwarfing in wheat that fueled the Green Revolution. To date, no commercial crops in which TFs have been modulated have been released; however, a major focus of research is on TF-mediated

resistance to abiotic stress, such as drought tolerance for which several candidate TFs have been identified in different crops.

The Safety Assessment of Crops Developed Using Gene-Modulation

Since small RNAs and TFs comprise a significant portion of the plant genome and humans and animals routinely consume plants, they are exposed to DNA, RNA, and in the case of TFs, small quantities of TF proteins in their diets. No adverse effect has ever been reported from the ingestion of DNA or RNA. Products such as the FLAVR-SAVR tomato and virus-resistant vegetables were approved because DNA and RNA are safe to consume, and no novel proteins were produced in them. Medical and pharmaceutical uses for small RNAs are an active focus of investigation; however, a major stumbling block has been natural barriers found in vertebrates to the uptake of preformed nucleic acids. Small RNAs do not appear to be active by an oral route of administration unless they are encapsulated in an invasive bacterium that transports them into the bloodstream like a Trojan Horse.

As noted above, crops developed using small RNA-mediated mechanisms produce no novel proteins and would therefore be exempt from protein safety assessment. TFs are proteins; however, they are present in very small amounts since they are produced in very few copies per cell. There is also a long history of consuming TFs without any reported adverse effect. Most proteins are innocuous⁴; no characterized TF has any similarity to a known allergen, toxin, or anti-nutrient. It is likely that a TF that would be employed in genetic engineering of a plant would be from that plant, or perhaps from a closely related crop plant with a

high likelihood that it has been encountered previously in the diet. Considering the above, and the fact that plant breeders have altered small RNA and TF content for millennia, it seems reasonable to conclude that modulation of small RNA species or TF production *per se* is not likely to produce new food safety risks.

The most significant changes that might be encountered in crop plants developed through the use of gene modulation techniques is the elimination of a metabolite or the alteration of the composition of a plant. TFs in particular can change the timing and amount of gene expression, but are limited to the genes present in the plant. Thus, they cannot result in the production of novel compounds; they can only alter relative amounts of endogenous compounds. Thus, Parrott et al. (2010) conclude that the safety assessment paradigm used to assure the safety of genetically engineered crops appears to be more than sufficiently robust for application to gene-modulated crops.

Since conventional crops with altered composition are not subjected to close regulatory scrutiny, it is hard to justify calling for careful compositional studies of gene-modulated crops from a scientific perspective. Such studies will doubtless be required by most regulatory authorities if for no other reason than to assuage misplaced concerns about the safety of genetically engineered crops. In such cases, the appropriate comparator is the conventional crop grown, to the extent possible, in conditions such as the transgenic version is designed to withstand (e.g., cold, drought). Thus, Parrott et al. (2010) conclude that the safety assessment paradigm used to assure the safety of genetically engineered crops appears to be more than sufficiently robust for application to gene-modulated crops.

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Large-scale Molecular Farming of Recombinant Human Collagen in Transgenic Tobacco

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Collagen and gelatin in medicine, drug delivery and cosmetics

Through provision of unmatched structural integrity together with cell and substrate anchorage ports, collagen fibers of the extracellular matrix represent macromolecules critical to embryonic development, tissue regeneration, and continual physical support of vertebrates and other multicellular organisms¹. Type I collagen, the prototype of fibrillar collagens, is the most ubiquitous collagen species in bone and tendon, and is found in significant quantities in skin, aorta, and lung, where variations in fiber diameter, orientation, and packing density dictate the mechanical properties of each tissue. Its primary role in skin structure and support is clearly highlighted upon loss of skin elasticity and strength as a consequence of age-related collagen degradation or crosslinking. Additionally, throughout the multi-stage wound healing process, collagen and collagen-derived fragments provide indispensable support for cell aggregation and adhesion, clot formation, fibroblast recruitment, and adequate scar tissue generation. Biocompatible collagen-based wound dressings or tissue substitutes contribute local haemostatic and chemotactic stimuli, while supplying a structural support upon which neotissue can be formed at enhanced rates. In addition, the highly absorptive character of such products accommodates the high exudate volume characteristic of injured tissue, providing a dry wound bed.

The gelatinous form of collagen, comprised of a mixture of irreversibly denatured collagen chains, features characteristics suitable for applications in food and beverage, pharmaceutical, photographic, cosmetic, paper manufacturing, and printing disciplines. Gelatins are used as binding, microencapsulation, and coating agents for pharmaceutical or health supplement preparations, and owing to their unique breakdown, can also be used to ensure slow release of active ingredients. In addition, their emulsifying effect can easily be manipulated by concentration and

temperature. Gelatin sponges and films are marketed as water-insoluble, absorbable medical supplies tailored to control bleeding and offer scaffolding support during early tissue regeneration processes. Expanding appreciation for the advantageous potential of collagen and its byproducts in technologies designed to restore dental, orthopedic, and cosmetic impairments is evident when considering the number of applications being introduced to the market.

Multiple post-translational modifications of collagen: a challenge to recombinant protein expression systems

The type I collagen heterotrimer is composed of two alpha 1 and one alpha 2 chains, constructed from repeating Gly-X-Y triplets, where X and Y can represent any amino acid but are typically proline and hydroxyproline². The polypeptide chains assemble to form a procollagen molecule within the rough endoplasmic reticulum (ER) assisted by the globular C-terminal extension propeptides, forming a trimeric molecule. The complex then folds in a C-to-N direction to yield a triple helix.

Procollagen biosynthesis involves a number of co- and post-translational modifications, including proline and lysine hydroxylation, glycosylation, and disulfide bond formation, all essential for the assembly and physiological stability of the final triple helix conformation. The enzymes responsible for these modifications act in a coordinated fashion to ensure appropriate folding and assembly of a correctly aligned and thermally stable triple-helical molecule.

Stability of collagen's triple-helical structure in mammals requires prolyl-4-hydroxylase (P4H) activity to form hydroxyproline residues within the collagen chains. Although plants are capable of synthesizing hydroxyproline-containing proteins, plant-derived prolyl hydroxylase exhibits relatively loose substrate sequence specificity in comparison to mammalian P4H³. Coexpression of collagen and

mammalian-derived prolyl-hydroxylase in insect⁴, yeast^{5,6} and plant⁷ cells supports the formation of stable, hydroxylated collagen.

Further posttranslational modifications of collagens involve the lysyl hydroxylase, galactosyltransferase and glucosyltransferase enzymes, which sequentially modify lysyl residues to hydroxylysyl, galactosylhydroxylysyl, and glucosylgalactosyl hydroxylysyl, respectively. These lysyl carbohydrate structures are unique to collagen and have been implicated in the control of fibril diameter⁸. The human Lysyl hydroxylase 3 (LH3) enzyme can consecutively catalyze all three modification steps required for hydroxylysine-linked carbohydrate formation⁹. In contrast, amino acid analysis of tobacco-expressed human collagen⁷ demonstrated hydroxylysine content to be less than 2% of that found in bovine collagen, suggesting that endogenic plant lysyl hydroxylase is unable to sufficiently hydroxylate collagen lysines.

Expression of Type I collagen in transgenic systems

Historically, collagen products used for pharmaceutical or biotechnological applications have been extracted from animal or cadaver sources. However, use of such materials can provoke immune responses and involves risk of contamination with pathogens.

Alternatively, bacterial and yeast expression systems are inexpensive and appropriate for certain proteins, yet often lack modifying enzymes and molecules required by complex proteins to reach full maturity. The complex biosynthesis of collagen, involving a relatively large number of enzymes managing its expression and maturation, imposes considerable demands on expression systems. As P4H is essential to protein stability, the ideal model must express mammalian variants of the enzyme in compartments that will ensure collagen-enzyme interactions.

The absence of enzymes and co-factors necessary for proline and lysine hydroxylation in traditional

microbial expression systems, as well as lack of disulfide bridge formation in microbial cytoplasm, make them unfit for expression of functional collagen. Using a yeast-based fermentation system, FibroGen Inc. has developed recombinant human collagen type III and gelatins applied toward a gamut of pharmaceutical and medical device applications. In parallel, mammalian cell lines have been proven effective in induction of procollagen expression and secretion, yet require mass culturing volumes, costly nutrient supplementation, and extensive time-to-product periods, while under constant threat of sample contamination by host pathogens. High yields of fully mature collagen are secreted in the

milk of transgenic animals bearing mammary gland-targeting genomic collagen- and P4H-encoding inserts. However, the extensive development costs of such models limit significant progress of this expression system. Because protein synthesis pathways are highly conserved between plant and eukaryote systems, the plant can often effectively support expression of complex eukaryotic proteins.

“The advent of plant-made-pharmaceuticals involving genetic manipulations, programming plants to express molecules of therapeutic value, has introduced a feasible alternative to conventional, fermentation-based expression models.”

Molecular farming in transgenic plants: a new industry

The recent biotechnology boom has the potential to introduce a wealth of pharmaceutical products and devices to the healthcare market. However, the costly infrastructure and limited production capacity associated with

expression of recombinant molecules via microbial fermentation or mammalian cell expression systems hinder realization of the majority of these potential products. The advent of plant-made-pharmaceuticals involving genetic manipulations, programming plants to express molecules of therapeutic value, has introduced a feasible alternative to conventional, fermentation-based expression models. Plant engineering offers cost-effective, safe, manipulable, and easily scalable protein yields harvestable after culture periods significantly shorter than in other expression systems.

While biopharming has sparked much debate over concerns relating to food chain contamination, gene

flow, and quality control guidelines regarding fungal toxins and pesticides, tight regulatory supervision has led to significant promotion of this discipline, which is critical to researchers, patients, and farmers. The absence of human and animal pathogens in plants offers an added feature to the use of such systems. The risk-benefit ratios are further enhanced when considering the tobacco plant as a model for production of biologics at commercial-scale levels. As a non-food crop with a large leaf mass and prematurity stage harvesting, both concerns of food supply contamination and of gene flow can be avoided. The decline of traditional tobacco agriculture followed by the search for novel farming opportunities has prompted the harnessing of tobacco plant production capacity toward meeting the growing demand for biologics.

Plant nuclei and chloroplasts alike have been exploited for high-yield expression of proteins, biopharmaceuticals, antibodies, and vaccines that demonstrate structural and posttranslational specifications closely resembling those of their natural counterparts. However, lack of specific enzymatic support often leads to plant-derived recombinant molecules void of modifications critical to their half-life and activity. Subcellular targeting of recombinant protein expression represents an additional factor influencing the maturity and yield of biologically relevant products expressed in the plant¹¹.

Collplant Ltd. reported the integration of a matrix of distinct plant features toward development of a high output system for expression of hydroxylated, heterotrimeric, recombinant human procollagen type I (rhPCOL1).

Through a series of crossbreedings, a tobacco line was engineered to coexpress vacuole-targeted human procollagen alpha 1 and alpha 2 chains together with human posttranslational modifying enzymes P4H alpha, P4H beta, and LH3. The final product, expressed in a compartment free of homologous plant enzymes or potential P4H inhibitors, proved viable and active¹². The purified and processed heterotrimeric recombinant human collagen (Collage rhTM) product is thermally stable, demonstrates fibril-forming capacities, and supports the attachment and expansion of various primary human cells normally involved in tissue repair processes (Fig. 1).

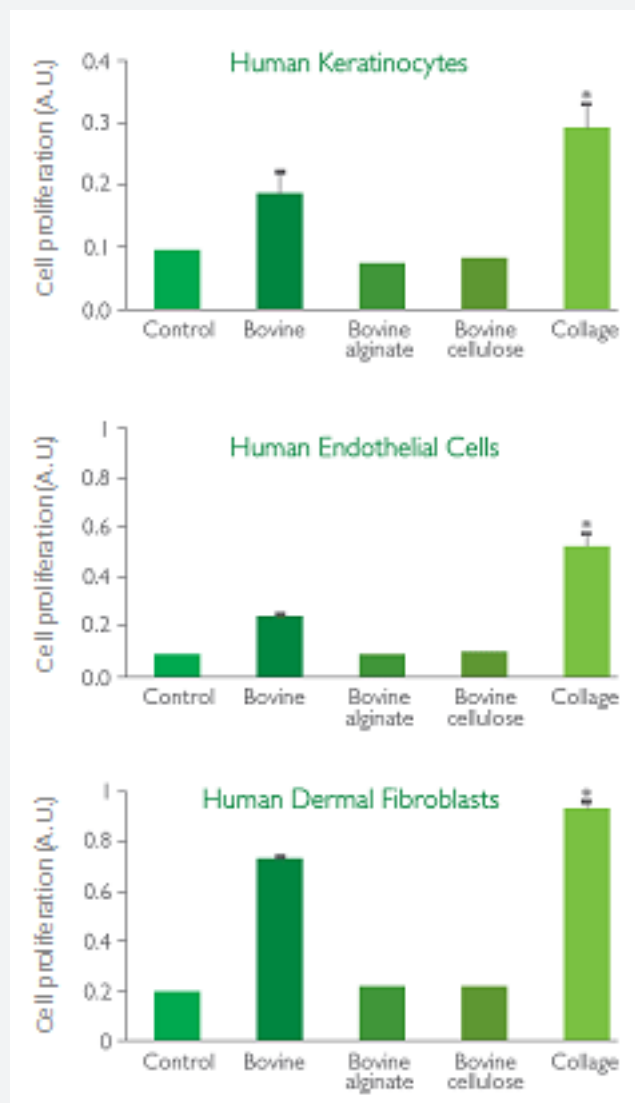


Figure 1. Enhanced cell proliferation on Collage rhTM scaffolds

Primary human keratinocytes, endothelial cells and dermal fibroblasts (25×10^3 cells/well) were seeded in on 6 mm diameter scaffolds that were placed in 96-well tissue culture plates. The scaffolds used were composed of bovine collagen, bovine alginate, bovine cellulose or recombinant human collagen (Collage rhTM). Negative control samples included cell media only or scaffolds in media, with no cells. Cells were allowed to proliferate on scaffolds for 72 h (37 °C, 5% CO₂). Scaffolds were then transferred to clean wells, supplemented with fresh media and WST-1 proliferation/viability reagent (1 hr, 37 °C, 5% CO₂). Absorbance, indicative of viable cells, was determined (450 nm) using and ELISA plate reader. Means of triplicate samples are presented (+/-SD). * Student's t-test: p<0.05. (Source: *Large-scale molecular farming of recombinant human collagen in transgenic tobacco*, Oded Shoseyov et al.)

Conclusions

Novel biopharming ventures feature a multitude of revolutionary prospects. Increased awareness of the plant-based protein expression system, farmer education, and appropriate regulatory policies and measures will boost full exploitation of these highly manipulable and cost-effective natural bioreactors.

More specifically, affirmative actions taken to facilitate such agricultural and technological opportunities can reverse the unfavorable public image of tobacco. Such measures will stimulate exploitation of the tremendous protein production advantages innate to the tobacco plant and will reestablish its credibility as a legitimate crop with the potential of benefiting mankind.

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