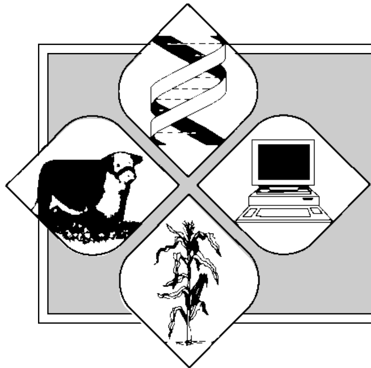

ISB NEWS REPORT

COVERING AGRICULTURAL AND ENVIRONMENTAL BIOTECHNOLOGY DEVELOPMENTS

FEBRUARY 2001

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NEWS AND NOTES

USDA ANNOUNCES RISK ASSESSMENT RESEARCH GRANTS

The Agricultural Research Service (ARS) and the Cooperative State Research, Education, and Extension Service (CSREES) are seeking proposals for the Biotechnology Risk Assessment Research Grants Program for fiscal year 2001. Research funded through this program will be relevant to risk assessment and the regulatory process. The purpose of the Program is to assist Federal regulatory agencies in making science-based decisions about the effects of introducing into the environment genetically modified organisms, including plants, microorganisms, arthropods, fish, birds, mammals, and other animals excluding humans. Investigations of effects on both managed and natural environments are relevant.

Proposals addressing the following topics are requested:

1. Research relevant to assessing the effects of the introduction into the environment of genetically engineered organisms.

2. Research on large-scale deployment of genetically engineered organisms, especially commercial uses of such organisms, with special reference to considerations that may not be revealed through small-scale evaluations and tests and may address cumulative effect concerns.

3. Research to assess the effects of transgenes in wild relatives of crop species, including research to evaluate the potential for unexpected fitness effects.

4. Research to assess the effects of genetically engineered plants with "stacked" resistance genes or genes that confer broad resistance to insects or diseases that may give recipient plants a greater selective advantage and lead to less predictable ecological consequences.

5. Research to develop statistical methodology and quantitative measures of risks associated with field testing of genetically modified organisms.

All proposals must be received at USDA on or before March 15, 2001. For supplementary information, please consult the full text of this Solicitation accessible at http://www.reeusda.gov/crgam/biotechrisk/biotech.htm.

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THE ISB NEWS REPORT

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CLONED GAUR DIES

Days after making history as the first interspecies cloned animal born, the cloned gaur died from common dysentery. The wild Asian ox or gaur is an endangered species that was cloned to evaluate cloning as a method to save a species from extinction. Cells from a gaur bull were fused with enucleated oocytes from domestic cows. Twelve percent of the reconstructed oocytes developed into blastocysts and 18% of these blastocysts developed into fetuses after transfer to surrogate cows. Examination of fetuses electively removed at 46 to 54 days of gestation or spontaneously aborted at day 202 of gestation showed no gross abnormalities. Furthermore, molecular analysis revealed that the fetuses contained the expected gaur nuclear DNA and cow mitochondrial DNA. Although the death of the cloned gaur was a disappointment, this birth did show that interspecies nuclear transfer was successful and may represent a possible strategy to save endangered species.

Source

Lanza RP, et al. 2000. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* 2: 79-90.

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TECHNOLOGY NEWS

EMERGING TECHNOLOGIES IN PLANT BIOTECHNOLOGY

The agricultural biotech industry has yet to supply a genetically modified (GM) product that is viewed as entirely beneficial by a largely skeptical public. Sections of the community perceive the industry as profit-driven, largely unaccountable, and interested in solving the problems faced by large-scale agricultural industrialists rather than those of the public at large. The ability to bring together traits from different plants, even from animals and microorganisms, concerns many people, and their belief is that such power should be wielded responsibly or not at all.

Traditional crop biotechnology typically involves the transformation of a human food plant with a foreign gene(s) coupled to plant virus

DNA and a herbicide/antibiotic resistance gene. The whole process is rightly seen as an extraordinary undertaking, requiring extraordinarily good justifications. The biotech industry has yet to convince skeptics that there are sufficiently extraordinary reasons for most of the products in or approaching the market. The lack of perfect knowledge and consequent unpredictability of the outcome of genetic manipulations further increase the anxiety and concerns about safety and the long-term consequences of GM products.

The first phase of plant GM technology has involved the random insertion of whole genes controlled by viral promoters into plants, and the widespread use of antibiotic/herbicide resistance genes to allow transgene selection. In the near future, these methods will likely be seen as primitive, clumsy, and perceivably risky approaches for creating novel crops with beneficial properties. To date, these techniques have provided very powerful research and development tools and are currently the only way to create truly novel products, such as plants that make biodegradable plastics in cotton fiber for a warmer product[1]. The emerging next phase of the technology addresses some of the first phase problems by, for example, using efficient and precise insertion of transgenes into the maternally inherited (at least in most plant species) plastid genome to allow a more controlled effect on plant metabolism. In most crop species, positioning the transgene in the plastid genome prevents its inclusion in pollen and therefore ensures genetic containment and avoids distribution of transgenes to honey products and pollen feeders, another public concern. It is now possible to also remove marker genes once they have been used in the initial identification of transgenic plants[2], a very positive development in the technology. (See "Engineered Chloroplasts Snip Out Antibiotic Resistance Genes," ISB *News Report*, this issue.)

In addition to the introduction of novel or altered genes into a plant, many metabolic advantages and new products can be gained by switching off plant genes using antisense or partial sense technology. This first phase technology uses a manipulated version of the target gene to control the expression of the plant's own version. This technique brings with it all of the problems already discussed. With the flood of information coming from functional genomics[3], proteomics, and protein structural studies, it is clear that genetic engineering can now incorporate rational design strategies and precision manipulation of heritable material to both knockout and change the characteristics of encoded enzymes.

The task is now to identify ways to create novel plant traits with a minimum amount of change since, armed with this new knowledge, a handful of carefully chosen tiny alterations (in many cases just of one base pair) can often have the required effect[4]. Minimizing the degree of genetic alterations is much more palatable to the public than the insertion or deletion of large amounts of coding DNA, with the same end result. This approach would benefit the biotech industry and may increase public support for more ambitious genetic engineering projects with widely beneficial goals. Some research effort should therefore be directed toward the development of tools that allow such effective but minimal genetic manipulations. Surprisingly, until recently the biotech industry has largely tended to reject the exploitation of single point mutations or polymorphisms.

A recent publication by Berns and co-workers[5] demonstrates a method that may one day replace the large-scale genetic tampering currently employed. The DNA of dividing animal cells in culture was made photosensitive by the addition of a dye, and a laser was aimed at a single visually identified region of a chromosome. The laser beam then knocked out, in a specific manner, the cluster of genes known to be located in that region. A heritable genetic modification had occurred without the use of any recombinant DNA. The problem is one of specificity; how does one direct the laser energy to only the specific gene or regulatory element one wishes to change? One possible answer is mentioned in the report's conclusion: utilizing the plants own homologous recombination system to precisely deliver a sequence-specific molecular probe conjugated to a photon-absorbing molecule. The delivery of such probes to the genes of cultured cells is entirely feasible, and this negates the requirement for microscopic aiming of the laser. Such an approach would allow the delivery of sufficient energy to a precise location in the genome, perhaps disrupting a specific regulatory element and causing a cascade of effects predicted from genomic/proteomic information. An example use might be the knocking out of a crucial region of the promoter for a gene encoding a transcription factor required for senescence, postponing flower decay.

The first use of another powerful site-specific mutagenesis technique in plants was reported last year[6]. Unlike laser mutagenesis, chimeraplasty is capable of introducing precise single point mutations into the genomes of cultured cells. In this case, however, recombinant heritable material is used. DNA/RNA hybrid oligonucleotides are introduced using standard techniques

(electroporation, biolistics) into cultured cells where they bind specifically to the DNA region of interest. A single 'error' in the oligonucleotide causes the cell's own DNA repair mechanisms to rewrite the DNA sequence to incorporate the single change. In the report, maize plants gained resistance to a common herbicide via a single change in the gene encoding for a protein involved in amino acid synthesis. The change was predicted to have no other effect on the metabolism of the plant. This is another example of a single precisely delineated alteration having a dramatic and useful effect. Herbicide resistance was used merely to identify altered plants easily, but information from functional genomics studies could identify many other targets with more beneficial outcomes.

In summary, techniques currently in development, when coupled with the enormous amounts of information coming from genomic and proteomic efforts in industry and academia, will stimulate a change in the way targeted genetic modification is employed. The precision of the new methods should address at least some of the public's concerns and help them to comprehend the benefits of agricultural biotechnology.

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Plant Research

Engineered Chloroplasts Snip Out Antibiotic Resistance Genes

A novel clean gene transformation technology that eliminates antibiotic resistance genes from transgenic chloroplasts has been developed in the UK.

Despite the potential of genetically modified (GM) plants for improving the quantity and quality of crops, they have not received the worldwide consumer acceptance that might have been expected. GM-antagonists argue that the widespread use of transgenic plants poses an unacceptable risk to the environment and human health. Whilst the strength of this anxiety over GM technology is difficult to justify, it has meant that transgenic plants themselves and the technologies used to generate them are facing ever-increasing scrutiny. The presence of antibiotic resistance genes in GM crops and their dissemination via pollen are particularly controversial issues. In the UK, a subgroup of the Advisory Committee on Releases to the Environment (ACRE) has been given the specific task of examining emerging technologies that could contribute to the development of transgenic plants designed to minimize environmental exposure to transgenes and their products (http://www.environment.detr.gov.uk/acre/bestprac/index.htm).

Antibiotic resistance genes are common components of gene transfer technologies. Transgenic plants are usually made by transferring one or two genes, commonly from another plant or bacterium, into chromosomes located in the nucleus of plant cells. Once transferred, these foreign genes are inherited along with the 25,000 to 50,000 native genes present on plant chromosomes. Gene transfer methods are inefficient and only a tiny proportion of cells usually take up foreign genes. To identify cells that take up foreign DNA, a foreign marker gene that confers a selectable property is required. These selectable marker genes are added alongside genes of interest. Antibiotic resistance genes are one of the most commonly used marker genes. Only plant cells that take up foreign genes proliferate in the presence of an antibiotic that kills unmodified cells. Once these transgenic plants have been selected, antibiotic resistance genes are no longer required, but they are usually retained. Whilst the antibiotics widely used to select GM plants are of limited oral use and the rate of transfer of antibiotic resistance genes from plant DNA to gut bacteria appears to be low (preventing its measurement), the presence of superfluous genes in GM plants is

increasingly viewed as undesirable. A report (December 1998) by a House of Lords select committee on the risks to human health of eating GM crops suggests that antibiotic resistance genes should be phased out of GM crops as swiftly as possible since alternatives are available (http://www.parliament.the-stationery-office.co.uk/pa/ld199899/ldselect/ldeucom/11/8121501.htm).

Chloroplasts are a suitable site for locating foreign genes

Plant cells contain DNA in three subcellular compartments. Approximately 80% of the DNA is located in the nucleus as chromosomes, 10-20% in chloroplasts, and around 1% in mitochondria. There are approximately 10-100 chloroplasts per cell. The DNA present in chloroplasts is circular and contains about 100 genes. Around 500 to 10,000 copies of these chloroplast DNA circles are present per cell. Methods to introduce foreign genes into chloroplasts lagged behind those used to insert foreign genes into plant nuclei. The stable integration of foreign DNA into chloroplasts was first demonstrated in 1988 using *Chlamydomonas*, a unicellular green alga, and particle bombardment technology developed at Cornell University[1]. Microscopic tungsten particles coated with foreign DNA were accelerated into target cells. The particles penetrated the tough cell walls and delivered foreign DNA into chloroplasts. This success in *Chlamydomonas* was repeated in tobacco[1]. Procedures based on bathing plant cells in media containing foreign DNA and polyethylene glycol have also allowed foreign genes to be introduced into chloroplasts[1].

The chloroplast is a suitable location for a wide range of foreign genes including those involved in photosynthesis, starch synthesis, fatty acid synthesis, oxidative stress tolerance, and those conferring tolerance to herbicides. Moreover, chloroplasts are useful compartments for storing polymers and pharmaceuticals. Although all foreign genes that we might wish to introduce into plants cannot be localized to chloroplasts, many of the genes that are perceived as "high risk" with respect to environmental impact, such as herbicide[2] and insect resistance genes[3], are functional in chloroplasts.

The insertion of genes into chloroplast DNA exhibits a number of desirable features. Since chloroplast DNA is small, it has been characterized in many plant species. The complete sequences of 18 different chloroplast genomes are known. Moreover, the gene content and organization of chloroplast DNA does not vary greatly between related species. This means we have detailed knowledge of the genomic environment into which we are inserting foreign genes, and the insertion of foreign genes into chloroplast DNA can be controlled with complete precision. Foreign genes can be targeted to specific predetermined sites in chloroplast DNA using the native homologous DNA recombination machinery present in chloroplasts. Foreign genes are usually propagated in bacteria by linking them to vector sequences, which allow their replication in bacteria before they are integrated into plant DNA. Vector sequences usually remain attached to foreign genes when they are transferred into plant cells. Although the overall risk of gene transfer from GM plant to bacteria is small, removal of vector sequences will reduce this risk further by deleting sequences that are known to promote replication in bacteria. When foreign genes integrate into chloroplast DNA by homologous recombination, bacterial vector sequences are excluded and are not present in genetically modified chloroplast genomes.

The release of pollen from many GM crops is a major route for transgene dispersal into the environment. Unlike nuclear chromosomal genes, which are transmitted in equal proportions by egg and sperm cells, chloroplast genes are transmitted solely via egg cells in many crops. This maternal pattern of inheritance prevents the pollen-mediated spread of foreign genes located in chloroplasts and is of considerable benefit for the environmental containment of transgenes. When two chloroplast types are present in the same plant, they tend to sort out into two pure populations of each chloroplast type. This cytoplasmic sorting process is unique to genes located in chloroplasts and mitochondria. Maternal inheritance, cytoplasmic sorting, and the rarity of DNA exchange between two chloroplast types, reduce, if not eliminate, the possibility of stacking chloroplast types resistant to different herbicides.

Efficient transformation of chloroplasts has relied on the *aadA* marker gene[4] that confers resistance to the antibiotics streptomycin and spectinomycin. Because there are hundreds and in many cases thousands of copies of chloroplast DNA per cell, the introduction of foreign genes into chloroplasts is a two-step process. In the first step, the *aadA* gene integrates into a fraction of the chloroplast DNA molecules present in a cell. In the second step, modified chloroplast genomes containing *aadA* are selected with spectinomycin and streptomycin until they replace all wildtype chloroplast genomes after repeated cell and chloroplast divisions. Once a plant is homoplasmic, that is contains only modified chloroplast genomes, the *aadA* gene is no longer required.

Removing antibiotic genes from chloroplasts[5]

Removal of *aadA* from chloroplasts is desirable. Although spectinomycin and streptomycin are rarely used clinically and not dispensed in the community, streptomycin is used occasionally for treatment of resistant *Mycobacterium*

tuberculosis in hospitals. The task of removing *aadA* from chloroplast DNA presents a challenge since it requires *aadA* excision from many copies of chloroplast DNA per cell. We decided to utilize the homologous DNA recombination machinery in chloroplasts to excise *aadA* genes rather than adding foreign recombinases and their target sites, such as the Cre/lox system, that have been used to excise antibiotic resistance genes from nuclear chromosomes. Our approach obviates the subsequent need to remove foreign recombinase genes from GM plants.

We used short direct DNA repeats to confer instability to the *aadA* gene. The degree of instability was important. If loss of *aadA* was too high, we would have been unable to use *aadA* to select plants containing modified chloroplast genomes. Correspondingly, a low frequency of *aadA* loss would have prevented us from isolating *aadA*-free plants. We found the correct level of *aadA* instability by increasing the number of short direct repeats in a construct from two to three. Once we were able to accumulate a high proportion of *aadA*-free chloroplast genomes within a plant, we relied on cytoplasmic sorting to isolate *aadA*-free tobacco plants. The *aadA*-free plants we isolated either contained a *uidA* reporter gene or herbicide resistance gene located in chloroplasts.

This method will allow a large range of genes, for example those encoding pharmaceutical proteins and insect resistance, to be introduced into chloroplasts without antibiotic resistance genes. Although the procedure was developed in tobacco, it is likely to work in a range of plant species. The small number of species amenable to stable chloroplast transformation is a current limitation. The combination of maternal inheritance, precise gene targeting, and removal of vector and antibiotic resistance genes makes the chloroplast a favorable site for locating transgenes in order to reduce their environmental impact. These positive features should accelerate the pace of research on chloroplast transformation of major crops.

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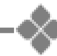

The Vision of an Edible Vaccine for Hepatitis B Starts to Come into Focus

Over two billion people worldwide are infected with hepatitis B, a serious liver infection that can result in jaundice, cirrhosis, and liver cancer. Although an injectable vaccine now exists, its expense and requirement for refrigeration makes it unavailable to more than one-third of the world's population, especially in poor countries where the vaccine is in urgent need. Dr. Charles Arntzen, of Arizona State University, has put the idea of a stable, plant-based vaccine forward as an attractive alternative. Now Dr. Arntzen and his colleagues report encouraging results in their effort to create a stable, edible form of the vaccine in their November 2000, *Nature Biotechnology* progress report.

Traditional vaccines rely on challenging the body's immune system with a weakened or killed form of a pathogen. The immune system "remembers" the chemical characteristics of the pathogen and responds quickly and effectively when exposed to attack by the fully potent form of the organism. More recently, vaccines consisting of a single component of the pathogen, such as the coat protein of a virus, have been equally effective. The current, conventional hepatitis B vaccine utilizes a single protein, HBsAg, produced in yeast, that, when polymerized correctly, forms a complex mimicking the structure of the actual virus. When injected, this complex triggers the body's immune system and provides protection from the disease.

The current hepatitis vaccine extracted from yeast requires chemical modification to become active, increasing the cost of the vaccine, which also must be stored under refrigeration. This has severely limited its utility in third world regions where the disease in rampant. The situation is further complicated by the need for three separate injections of the vaccine at 0, 1, and 6 months of age.

Edible vaccines have the potential to address many of the problems facing synthesis and distribution of vaccines. Plant-based vaccines can be grown locally, reducing the cost and complications of transportation, while the stability of proteins in intact plants removes the need for refrigeration. Furthermore, the edible nature of the vaccines eliminates the need for syringe-based delivery, saving money and reducing the risk of infections.

Although proof of the principle behind edible vaccines had been demonstrated by several groups in animals, it wasn't until 1997 that Arntzen and his colleagues, in collaboration with the University of Maryland's Department of Medicine, were able to test their ideas in humans[1]. A test group of 11 human subjects were fed raw potatoes engineered to express the B subunit of the *E. coli* toxin. Careful monitoring of the test subjects revealed that 10 of the 11 volunteers displayed a four-fold rise in serum antibodies to the toxin. Furthermore, six of the test subjects also displayed a corresponding rise in intestinal antibodies to the toxin. Further studies by Arntzen and other research groups have shown that systemic immune response is possible in humans using other plant-based vaccines.

Edible vaccines work in a very different manner than traditional vaccines. Since standard vaccines are injected parenterally, rather than administered orally, the detector elements of the immune system, the B and T cells, have direct access to large amounts of antigen. In contrast, orally administered vaccines are detected by components of the immune system known as M cells, which are present in the gut. These cells recognize the ingested antigen and transport it to the B and T cells, which in turn mount the immune response. Researchers, including Arntzen, have found that it takes a much higher dose of the antigen to provoke a full immune response when administered in this manner, possibly due to degradation of the antigen in the gut. Unfortunately, production of vaccine antigens in plants often fails to meet the minimum level required to produce an immune response when administered orally.

To overcome this limitation, Arntzen and his colleagues studied various ways to increase plant production of the hepatitis B antigen, HbsAg, in potato. The results of their efforts, along with further proof of the effectiveness of this form of the vaccine, were reported in the November 2000 issue of *Nature Biotechnology*[2]. One of the main concerns about using a plant-based method of administering a hepatitis vaccine has been that transmission of hepatitis is believed to be non-enteric (not through the digestive tract). Although Arntzen and his group had previously shown that the plant-produced form of HBsAg was capable of initiating an immune response when injected into mice, they still needed to establish that the protein contained in potato tissue could stimulate the same response when fed to mice. The ability of plant-produced HBsAg to trigger an immune response when administered orally had been previously established by Hilary Koprowski and his group at Thomas Jefferson University[3]. His studies found that HBsAg produced in lupine and lettuce was able to cause the production of HBsAg-specific antibodies when fed to mice and humans, respectively.

To test the immunogenicity of the potato-generated HBsAg, Arntzen and his fellow researchers used a line of potatoes that had been engineered to express the gene encoding HBsAg from the tuber-specific patatin promoter. This particular line of transgenic potatoes accumulated 1.1 µg of HBsAg per gram of fresh tuber. Mice were fed a total of 16.5 µg HBsAg over a course of three weeks, along with 30 µg (total) of cholera toxin (CT) to act as an adjuvant (general immune stimulant). Unlike the control mice fed only regular tubers and CT, the experimental mice displayed a primary serum antibody response that peaked at 73 mIU/ml three weeks following the last dose. Furthermore, a high-level recall response was observed only in the experimental mice upon injection of a subimmunogenic dose of commercial hepatitis vaccine, indicating that the feeding of transgenic tubers had established immune memory in the mice.

Although these results were encouraging, Arntzen and his fellow workers believed that a stronger immune response could be triggered if they were able to increase the amount of protein produced by the potato plants per gram of tuber. A number of factors influence the level of protein produced in transgenic plants. Modifying the promoter, 5'-untranslated, and 3'-polyadenylation signal regions has, in all cases, modified the expression of a transgene in plants. However, it has also been observed that the level of expression of an introduced gene varies between transgenic individuals carrying the same construct. This is thought to be due, in part, to the location within the genome of transgene insertion (positional effects) as well as the number of copies of the gene that insert (copy number). To account for any variation caused by these last two factors when conducting comparisons between different constructs, Arntzen devised a method of calculating the amount of protein produced per number of transcripts present for each of the expression constructs under study. To further control for any individual effects, data was collected from several individual transgenic plants representing each construct.

In an attempt to increase the expression of HBsAg in potato, Arntzen and his colleagues tested the introduction

of a number of signaling peptides and 5'- and 3'-untranslated regions (UTRs) in constructs driven by the nominally constitutive cauliflower mosaic virus (CaMV) 35S promoter. Sequences tested included 5'-UTRs from tobacco etch virus and tobacco mosaic virus, and 3'-UTRs from the soybean *vspB* and potato *pinII* genes. After normalizing to transcript levels as described above, Arntzen found that the use of different 5'-UTRs had little effect on expression levels, but the introduction of the *vspB* and *pinII* 3'-UTRs increased the amount of HBsAg protein significantly. Unfortunately, the highest expressing lines also exhibited stunted growth and reduced tuber formation, indicating that high levels of the introduced protein may be phytotoxic. Interestingly, the introduction of endoplasmic reticulum (ER) retention signals also increased the amount of HBsAg protein per unit mRNA. The authors theorized that retention of protein in ER might lead to increased subunit interaction, stabilizing the protein and protecting it from degradation.

The results reported by Arntzen and his group represent encouraging support of the concept of plant-delivered vaccines. However, there remain significant obstacles before an edible hepatitis B vaccine becomes a reality. The possible phytotoxicity of hepatitis antigen to the plant production source will have to be overcome. It may be possible to work around this obstacle through the use of adjuvant, possibly also expressed within the plant. This may make it possible for the body to detect and respond to a lower dose of antigen. As the research advances, the issue of dosage control will become significant—just as with standard vaccines, it will be critical to ensure that patients do not receive too much or too little of the antigen. On the more aesthetic side, it may be necessary to find a more palatable form of the vaccine, as the taste of raw potato is not particularly pleasing to many. Efforts are already underway to make banana-based edible vaccines. However, any form of stable, edible vaccine would be welcomed in many parts of the world. With the results of his recent paper, Dr. Arntzen has taken this vision one step closer to reality.

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Plants that Detect Landmines, and Other Biosensors

The furor over genetically modified (GM) plants has focused on crops with engineered agronomic qualities, such as insect resistance and herbicide tolerance. Critics are primarily concerned with food safety issues and ecological consequences. But GM technologies have much further ranging applications than simply agriculture. While their potential for improving crops plants is important, environmental monitoring is an area where GM plants can perform a crucial role in a low-impact manner, a role that even GM opposition would be hard-pressed to criticize.

It is possible that in the near future, GM plants could provide constant, landscape-level data on environmental hazards. Current monitoring of surface and sub-surface contaminants, both inorganics such as heavy metals and organic toxins such as PCBs, relies on frequent water sampling of wells, an expensive and labor-intensive task. GM plants could be used as biosensors to monitor radioisotope levels around nuclear power plants, or to detect jet fuel contaminants at military bases. They could also disclose the presence of certain other unwanted and especially dangerous substances in our environment of which presently there is no good way of monitoring. One of these is buried explosive devices.

Several laboratories are currently performing research using plants as biosensors. This entails the isolation of specific inducible promoters (gene switches) that can be fused to one or more genes encoding a visible marker. The current visible marker of choice is green fluorescent protein (GFP) from the Pacific jellyfish (*Aequorea victoria*). GFP has the unique property of fluorescing green when excited by blue or UV light. A DNA construct containing a specific inducible promoter could be fused to GFP and then transferred into a plant for biomonitoring. For example, in a case where the promoter has been isolated and modified so that it is responsive to copper[1], plants containing this construct might be deployed around the periphery of a copper mine to monitor copper movement into the surrounding countryside. When copper was present in sufficient concentrations to trip the promoter, plants would fluoresce green at that location. Ultimately biosensors of nature could be used in conjunction with bioremediators, the perfect marriage being that where an individual plant performs both functions.

The application we want to consider here is the detection of buried explosive devices, such as landmines. Landmines generally have small plastic housings of extremely inexpen-

sive construction that contain trinitrotoluene (TNT) or other explosives. Landmines come in various sizes and shapes and are, for the most part, designed to explode and maim whatever steps on the soil surface covering the mine. Cambodia, Angola, and Pakistan are examples of countries that are littered with landmines deployed by invading military, government, and/or rebel groups. Most of the nations in this situation are those whose economies rely heavily on non-automated agricultural production, and the presence of landmines effectively removes large areas of arable land from agricultural production. The more obvious and urgent problem is that these mines kill and maim multiple people everyday. Those who live in the vicinity of minefields are normally aware of the existence of the minefield itself, but not of the specific locations of the mines that may be planted randomly as little as 10 meters apart. Since they are plastic, landmines cannot be located by metal detectors; in the developing world, the most common de-mining practice is that of a man with a stick. He will search for a landmine by feel, a practice that is imprecise at best and often a hideous short-term career at its worst.

The idea to use organisms to detect TNT was first exemplified using GM bacteria by Dr. Robert Burlage and his coworkers at the Oak Ridge National Laboratory[2]. Their bacteria, *Pseudomonas putida*, had a TNT inducible promoter fused to GFP and were tested on a faux minefield with surrogate landmines. *Pseudomonas putida* detected five of five landmines in a one-quarter acre plot; however, they also produced two false positive signals, indicating the presence of a landmine where none existed. There are several other drawbacks to a bacterial-based system. It requires that bacteria be grown and sprayed onto the minefield, which could be determined to be environmentally unacceptable. A government also might well object to the release of recombinant bacteria in the interest of national security. Additionally, it has been found that the bacterial signal is dependent on a plant substrate for bacterial colonization. A plant-based detection system has the advantage of utilizing a macroscopic, and trackable, organism; TNT would be absorbed by plant roots and then transported to leaves[3] where the fluorescence could be readily observed. The root structures would also more effectively mine the soil for trace explosive, resulting in increased mapping accuracy. Another advantage is that the plants used could be optimized for specific ecological conditions.

How would such a plant-based TNT detection system work? The first step would be sowing detector plant seed over a minefield in a manner that would result in uniform coverage, a potential logistical problem that would require helicopter-based seed pelleting. A homogeneous stand of plants would need to be established so the roots could cover the mine-leachate soil volume. It would be necessary to supply plants with water and nutrients via a helicopter to assure they will be in good health with normal protein production. Finally, plants would need to be detected. Simply, the plants located over a landmine would fluoresce green; those not in the proximity of a mine would not fluoresce. The false positive signals in the bacterial system were due to leaching[2]; a plant-based system, where the promoter is tripped by an accumulation of TNT, should not be nearly as vulnerable.

An important component of any detection system is the photonic device used for picking-up the fluorescent biosensor signals. At our laboratory in North Carolina, we work at ground level in the dark using a strong UV lamp to shine on GFP-producing plants. Obviously, this practice is not transferable to a field situation where it becomes far too reminiscent of the man with the stick. In another National Laboratory in Santa Barbara, John Di Benedetto and his colleagues have produced laser-induced fluorescence imaging (LIFI) and laser-induced fluorescent spectroscopy (LIFS) devices that can be used to detect and measure fluorescence from a stand-off[4]. Scaling remote sensing to airborne devices is critical for the successful detection of biological-based landmine detection systems and other real-time biosensors. Says Dr. Di Benedetto, "(Airborne) laser-induced fluorescence will provide remote access and direct evidence of specific contamination, whether it is TNT, heavy metals, or pathogens. It will bring a whole new dimension to remote sensing."

The uses of biotechnology will increasingly move from the agricultural and medical toward environmental applications. A number of research groups in both the public and private sectors are working to make environmentally useful GM organisms a reality. We believe the wholesale environmental objection to GM plants will be moderated by landmine-detecting GM plants, as well as other explicitly environmentally useful GM plants. A biotechnology that can monitor the presence of environmental contaminants, and then possibly even clean them up, has the potential to replace expensive extant technologies. For example, plants that can bioremediate mercury have already been developed[5]. These plants have the ability to absorb and convert toxic forms of mercury to less-toxic elemental mercury that they subsequently volatilize, and a mercury-inducible GFP marker would be a useful addition. Biotechnology can and should play a pivotal role in monitoring toxins and in cleaning up the environment. A GM plant that detects the location of landmines seems like a biotechnological advance that even Prince Charles could love.

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Animal Research

Transgenic Milk Containing Lysostaphin: A Possible Cure for Mastitis?

Mastitis is an infection of the mammary gland that affects more than half of all US dairy cows and costs the dairy industry more than $180 per cow annually. This disease represents more than a billion dollar loss to the US dairy industry. One of the most common causes of contagious mastitis is an infection by the bacterium, *Staphylococcus aureus,* which causes tissue damage and decreased milk production and is difficult to control with the use of antibiotics.

In the January 2001 issue of *Nature Biotechnology*, researchers from the University of Vermont, the Institute for Animal Health in the UK, and the USDA Agricultural Research Service (Bethesda, MD) report on a possible biotechnological solution to the problem. This research team generated transgenic mice that secrete into milk the potent anti-staphylococcal protein, lysostaphin. Lysostaphin is a peptidoglycan hydrolase that is normally produced by *Staphylococcus simulans*. Transgenic mice expressing lysostaphin were found to exhibit substantial resistance to *S. aureus* infection.

Transgenic mice containing the lysostaphin gene were created using the standard method, microinjection of DNA into fertilized oocytes. However, the procedure was not as simple as taking the lysostaphin gene and injecting it into mouse oocytes. Preliminary studies showed that expression of the bacterial lysostaphin gene in eukaryotic cells resulted in the production of biologically inactive lysostaphin due to glycosylation of the protein. This necessitated the modification of the lysostaphin gene such that the amino acid sites for glycosylation were altered. Eukaryotic cells containing this genetically modified lysostaphin gene expressed the biologically active protein.

Eight founder transgenic mice were generated. Two of the eight did not transmit the lysostaphin transgene to their progeny. Three of the founder lines expressed lysostaphin in their milk at a very low concentration (<0.02 mg/ml). The other three founder mice expressed lysostaphin at varying levels: low (0.06 mg/ml), medium (0.13 mg/ml), and high (0.8-1.3 mg/ml). As expected, lysostaphin expression was almost completely restricted to the mammary gland because the mammary-specific, ovine beta-lactoglobulin promoter was used to regulate expression of the lysostaphin gene.

To evaluate their resistance to *S. aureus* infection, transgenic mice were challenged with an intramammary inoculation of 10,000 *S. aureus* particles. At this dose of *S. aureus*, all inoculated mammary glands of non-transgenic controls were infected. In contrast, in the high-lysostaphin producing line, glands were completely resistant to infection as were 40% of the glands from the medium and low lines. In addition, the infected glands from the control mice showed signs of tissue damage, whereas the infected glands from the transgenic mice showed normal morphology.

This study demonstrates that transgenic mice expressing the bacterial anti-staphylococcal protein lysostaphin show increased resistance to *S. aureus* infection. These transgenic mice appear normal in every way examined, such as physiology of the animal, fertility, integrity of the mammary gland, and profile of milk proteins. However, the resistance established would not provide protection against non-staphylococcal infections nor against infection during nonlactating periods. Nevertheless, this represents a very promising approach to controlling mastitis.

The next logical step is to generate transgenic dairy cows that express lysostaphin to see if these cows similarly show reduced susceptibility to *S. aureus* infection. Lysostaphin activity is specific for staphylococcal bacteria, so milk containing lysostaphin should not have any effect on bacterial cultures used in the dairy food industry. So milk, often considered the perfect food, may get even better.

Source

Kerr DE, et al. 2001. Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. *Nature Biotechnology* 19: 66-70.

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REGULATORY NEWS

PREMARKET NOTICE CONCERNING BIOENGINEERED FOODS

The US Food and Drug Administration (FDA) is proposing to require the submission to the agency of data and information regarding plant-derived bioengineered foods that would be consumed by humans or animals. FDA is proposing that this submission be made at least 120 days prior to the commercial distribution of such foods. FDA is taking this action to ensure that it has the appropriate amount of information about bioengineered foods to help to ensure that all market entry decisions by the industry are made consistently and in full compliance with the law. The proposed action will permit the agency to assess on an ongoing basis whether plant-derived bioengineered foods comply with the standards of the Federal Food, Drug, and Cosmetic Act. The proposed rule can be accessed at http://www.fda.gov/OHRMS/DOCKETS/98fr/011801A.htm

Comments on the proposal will be due by March 28, with comments on information collection provisions by February 17. Submit written comments to:
Dockets Management Branch (HFA-305)
Food and Drug Administration
5630 Fishers Lane, Rm. 1061
Rockville, MD 20852.

Submit written comments on the information collection provisions to:
Office of Information and Regulatory Affairs, OMB
New Executive Office Bldg.
725 17th St. NW., Rm. 10235
Washington, DC 20503
Attn: Desk Officer for FDA.

In a separate but related action, FDA is issuing a draft guidance document, which if finalized, would provide direction to manufacturers who wish to label their food products as being made with or without ingredients developed through biotechnology. A copy of the draft guidance on labeling bioengineered foods is available on the Internet at http://vm.cfsan.fda.gov/~dms/biolabgu.html. It may also be requested by calling 202-205-4561, or faxing a request to 202-205-4594.

For further information regarding human food issues, contact:
Linda Kahl
Center for Food Safety and Applied Nutrition
Tel: 202-418-3101.

For information regarding animal feed issues contact:
William Price
Center for Veterinary Medicine
Tel: 301-827-6652

IDENTITY PRESERVATION AND PRODUCT SEGREGATION PROCEDURES

As part of the Administration's biotechnology initiative announced last May, the United States Department of Agriculture (USDA) invites comments on how they can best facilitate the marketing of grains, oilseeds, fruits, vegetables, and nuts in a market that includes both crops derived from biotechnology and other crops. USDA is seeking comments on current and anticipated market practices, and on the feasibility of and need for USDA's involvement in quality assurance or other programs to facilitate the marketing of these products.

All interested persons are encouraged to comment on the issues related to this notice and to submit them to Richard Hardy by fax at 202-720-2459 or via the Internet at http://www.usda.gov/gipsa. Comments must be received on or before February 28, 2001.

For a detailed description of the issues and contact information, connect to: http://frwebgate.access.gpo.gov/cgi-bin/getdoc.cgi?dbname=2000_register&docid=00-30140-filed.

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