Agri-food research in Ontario / La recherche agro-alimentaire en Ontario



June 1996 - Vol. 19, No. 2

TABLE OF CONTENTS:

Cover and Forefront | Crop Improvement | Crop Protection | Animal Breeding | Animal Health | Food Safety | Improved Foods | Environment | More

COVER AND FOREFRONT:

- **Agri-Food Biotechnology: the Ontario Advantage**
- **Agri-Food Biotechnology: Unraveling the Mystery**

CROP IMPROVEMENT

- **The Advantages of Alfalfa Biotechnology**
- **Molecular Markers: Fast Track Screening for Crop Diversity, Desirable Traits**
- **Tran$formed Barley: A New Source of Mildew Resistance**
- **Genomic Mapping: Generating New Genes and Basic Science**
- **Transformed Cassava: Improving a Tropical Staple**
- **Micropropagation: Mass Production of Disease-free Horticultural Crops**
- **Doubled Haploid Technology Accelerates Canola Breeding**

| Table of Contents | Top of Document | Next |

CROP PROTECTION

- **Help for Horticulture Growers: Disease Diagnosis Quick & Sure**
- **PCR Diagnostics for Potatoes and Other Crops**
- **Engineering Pathogen Resistance to Potato Leafroll Virus**

- **Managing Aggressive *Trichoderma* in Mushrooms**
- **Hypovirulent Strains of Plant Pathogens: A New Pest Management Tool**

| Table of Contents | Top of Document | Next |

ANIMAL BREEDING

- **Integrating Biotechnologies into Genetic Improvement of Livestock**
- **Embryo Biotechnology for the Canadian Dairy Industry**
- **Cryobiology for Ontario Agriculture**
- **Reproductive Biotechnologies for Superior Ontario Swine Genetics**
- **Antibodies that Put Embryo Sexing on Target**
- **Transgenic Chickens: Promising Applications for Ontario's Poultry Industry**
- **Chickens as Bioreactors: Harvesting Commercially Valuable Proteins from the Egg**
- **Identifying Boar Taint Genes: Economic and Animal Welfare Benefits**
- **Stressed Pigs Get Better Fitting Genes**

| Table of Contents | Top of Document | Next |

ANIMAL HEALTH

- **High Immune Response Pigs - Healthier, Cheaper, Faster**
- **Inherently Healthier Holsteins**
- **Edible Vaccines - Transforming Alfalfa for TGEV Immunity**
- **Antibodies that Act as Hormones**
- **Battling Bovine Leukemia Virus**

| Table of Contents | Top of Document | Next |

FOOD SAFETY

- **Biotech Food Safety Diagnostics: Big Benefits, Big Business**
- **A DNA Hybridization Method for Isolation of Verotoxigenic *E. coli***
- **Toward Rapid On-Line Detection of Pathogens for Good Processing**

- **Epidemiologic Typing of Food-borne Bacteria**

| Table of Contents | Top of Document | Next |

IMPROVED FOODS

- **Biotechnology in Food Processing: Taste and Quality Top the List**
- **Genetically Engineered Wine Yeasts for Cooler Climates**
- **'Designer' Fats and Oils: Customized Lipids for Food and Pharmaceuticals**

| Table of Contents | Top of Document | Next |

ENVIRONMENT

- **Cleaning up Contaminants with Customized Microbes and Enzymes**

| Table of Contents | Top of Document | Next |

.....AND MORE...

- **Biotechnology at Work in Research**

| Table of Contents | Top of Document |

| Research, Education, Laboratories & Risk Management Home Page |

| Central Site | Feedback | Search | Site Map | Français |
| Home | What's New | Calendar | Products | News Releases |

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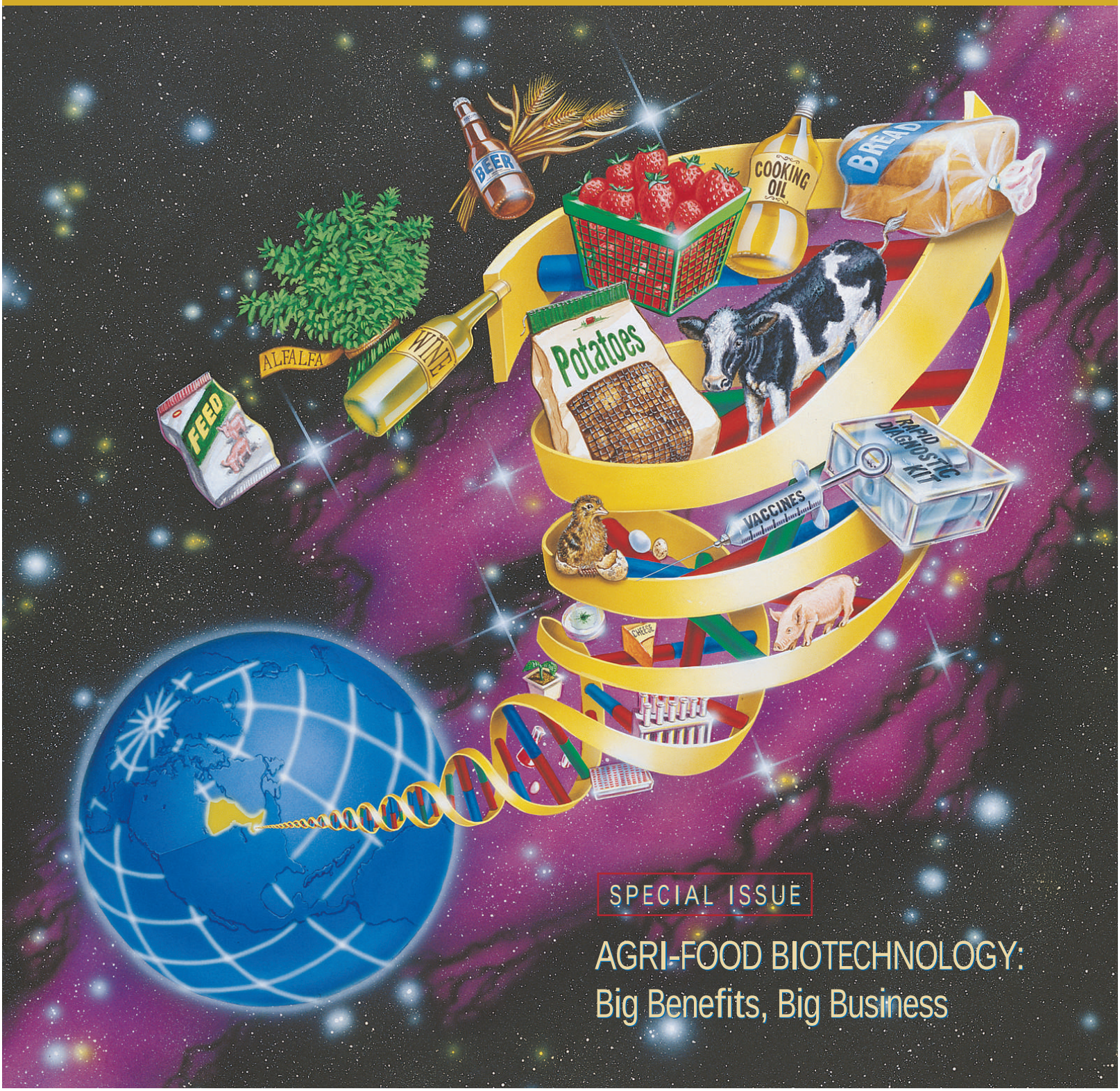
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JUNE 1996 VOL.19 NO.2

Ontario
Ministry of Agriculture, Food and Rural Affairs
Ministère de l'Agriculture, de l'Alimentation et des Affaires rurales

Agri-food research IN ONTARIO

La recherche agro-alimentaire EN ONTARIO



SPECIAL ISSUE

AGRI-FOOD BIOTECHNOLOGY: Big Benefits, Big Business

Agri-Food Biotechnology: the Ontario Advantage

When it comes to agri-food biotechnology, Ontario means opportunities and one stop shopping. That's not surprising – given Ontario's agricultural advantages: No. 1 in Canada in farm gate value; No. 1 in production diversity; food processing investment and exports. And all located at the centre of major Canadian and North American markets.

Then there's the wealth of technical expertise at six major universities linked with federal and provincial food, health, environment and agriculture centres at Ottawa and Guelph. A $30 million dollar research agreement between two key biotech players – the Ontario Ministry of Agriculture, Food & Rural Affairs, and the University of Guelph – highlight the collaboration that exists. No other university and government in Canada have such a direct research connection.



ILLUSTRATION: DOUGLAS J. DONALD

At the industry's Guelph Food Technology Centre, food marketing and processing challenges – many involving the tools of biotechnology – are solved collaboratively with scientists of every discipline, close by and ready.

The result is a highly integrated and successful industry - university - government network, with answers and expertise from product concept through to commercialization.

Here's just a few examples of the wide ranging, cooperative agri-food biotech research you'll find in Ontario:

- Food microbiologists working with dairy and meat processors to improve food safety.
- Crop scientists collaborating with food processors and feed companies to develop new transgenic plant varieties with improved quality traits.
- Animal scientists working with pharmaceutical and food companies to create new products for disease prevention.
- Food scientists working with food processors for consumer products with improved taste, freshness and nutrition.
- Pest management specialists developing new pathogen diagnostics with the horticulture industry for reduced crop disease and chemical control.
- Immunologists working with animal breeders to develop advanced vaccines and genetically healthier animals.
- Microbiologists developing environment-friendly microbes together with chemical companies for bioremediation of contaminants.

In Ontario, biotechnology doesn't stop in the lab. Support for industry continues through such efforts as GUARD, a for-profit, technology management company set up at the University of Guelph, to convert new technology into profitable business. GUARD is unique in Canada and established to ensure profits are returned to Canada.

Products, profit and public benefits, for the best of agri-food biotech ... the best place to look is Ontario.

For more information,
call 1-888-ONT-TECH.

Agri-food research IN ONTARIO

La recherche agro-alimentaire EN ONTARIO

JUNE 1996
VOL.19 NO.2

Formerly *Highlights of agricultural and food research in Ontario*

Contents



AGRI-FOOD BIOTECHNOLOGY: UNRAVELLING THE MYSTERY 1

Crop Improvement

The Advantages of Alfalfa Biotechnology 2
Molecular Markers: Fast Track Screening for Crop Diversity, Desireable Traits 7
Tran$formed Barley: A New Source of Mildew Resistance 9
Genomic Mapping: Generating New Genes and Basic Science 11
Transformed Cassava: Improving a Tropical Staple 14
Micropropagation: Mass Production of Disease-free Horticultural Crops 15
Doubled Haploid Technology Accelerates Canola Breeding 16



Crop Protection

Help for Horticulture Growers: Disease Diagnosis Quick & Sure **19**
PCR Diagnostics for Potatoes and Other Crops 21
Engineering Pathogen Resistance to Potato Leafroll Virus 23
Managing Aggressive *Trichoderma* in Mushrooms 25
Hypovirulent Strains of Plant Pathogens: A New Pest Management Tool 26

Animal Breeding

Integrating Biotechnologies into Genetic Improvement of Livestock 28

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Agri-food biotechnology offers unprecedented opportunities to improve food safety and processing, human and animal health, and crop and environmental protection.

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Contents



Embryo Biotechnology for the Canadian Dairy Industry 30
Cryobiology for Ontario Agriculture 32
Reproductive Biotechnologies for Superior Ontario Swine Genetics 34
Antibodies that Put Embryo Sexing on Target 35
Transgenic Chickens: Promising Applications for Ontario's Poultry Industry 36
Chickens as Bioreactors: Harvesting Commercially Valuable Proteins from the Egg 39
Identifying Boar Taint Genes: Economic and Animal Welfare Benefits 42
Stressed Pigs Get Better Fitting Genes 43

Animal Health



High Immune Response Pigs – Healthier, Cheaper, Faster 45
Inherently Healthier Holsteins 46
Edible Vaccines – Transforming Alfalfa for TGEV Immunity 47
Antibodies that Act as Hormones 48
Battling Bovine Leukemia Virus 50

Food Safety

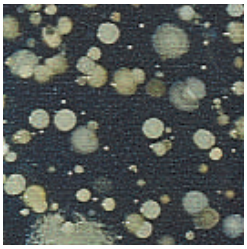


Biotech Food Safety Diagnostics: Big Benefits, Big Business 51
A DNA Hybridization Method for Isolation of Verotoxigenic E. coli 52
Toward Rapid On-Line Detection of Pathogens for Food Processing 53
Epidemiologic Typing of Food-borne Bacteria 55

Improved Foods



Biotechnology in Food Processing: Taste and Quality Top the List 56
Genetically Engineered Wine Yeasts for Cooler Climates 57
'Designer' Fats and Oils: Customized Lipids for Food and Pharmaceuticals 58



Environment

Cleaning up Contaminants with Customized Microbes and Enzymes 60

AND MORE... Biotechnology at Work in Research 62

Un resumé de chaque article est disponible en français, sur demande.

Agri-Food Biotechnology: Unravelling the Mystery

The term 'biotechnology' is widely used, but often little understood – and perceived with various opinions, depending on one's knowledge and point of view. As a broadly encompassing term, it simply means the use of biological organisms or systems (bio) to create new products or processes (technology). Humans have used living organisms and biological processes to produce food and drink for thousands of years – for example in bread and yogurt making, in fermentation of spirits, and in the production of cheeses.

Today, agri-food biotechnology offers unprecedented opportunities to improve food safety and processing, human and animal health, and crop and environmental protection, to name just a few.

These opportunities come from new biotechnology methods. For example, the transfer of selected genetic material from one organism to another can produce novel genetic traits and benefits not otherwise obtainable – advantages such as improved yields, better nutrition, stronger disease resistance, and more desireable consumer qualities.

But biotechnology in agriculture and food, or any sector, must be regarded in the proper context – as a tool that's most useful when it's integrated with other scientific and commercial processes. From a practical viewpoint, propheses of a quick panacea or of sudden doom are both misrepresentative. Many commercially desirable traits are too complex, governed by too many genes to be easily and quickly manipulated.

And research is costly – so that publicly or privately funded projects will only proceed in those areas where consumer demand exists. Progress will continue methodically, according to strictly estabished scientific protocols, in the direction that public support leads it.

Biotechnology is acknowledged to be "one of the high growth engines of the new knowledge-based economy"[1]. The public benefits of health, environment, and economy make it exciting for all Ontarians – from producers and processors to retailers and consumers. Read about some of the promising agri-food research underway here, on the following pages.



PHOTO: MASTERFILE, G. BISS

PHOTO: FPG INTERNATIONAL, MICHAEL SIMPSON

[1] From Enabling Biotechnology – a strategic plan for Ontario. Biotechnology Council of Ontario (1994).

The Advantages of ALfalfa

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Perennial forage crops are an essential part of Ontario's agriculture. They provide 60 to 85% of the nutrients fed to dairy and beef cattle and are the foundation of the sheep, goat, horse, and game farming industries. In addition to their feed value, forages conserve and improve Ontario's soil and the quality of its water. Of the total improved land in the province, 37% is used for forage crop production. Alfalfa, which is the most productive forage legume, is grown on most of this acreage.

Over the past 20 years, there have been tremendous gains in the genetic improvement of alfalfa for resistance to diseases such as Verticillium wilt, Phytophthora root rot, Anthracnose, Fusarium, and Bacterial wilt, and to insects such as the pea, spotted, and blue aphids. These genetic gains have contributed to longer stand life, greater yields, and reductions in production costs. The improvements in yield performance of alfalfa varieties, which require no significant increase in production input, are averaging 0.7% per year. This represents an increased value of $2.8 million per year in Ontario.

Superimposed on yield improvement have been genetic gains in feeding quality which can provide a net reduction in feed costs in mixed rations of approximately $15.25/cow/year.

Limitations of Conventional Breeding

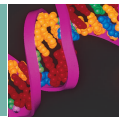
Our research has emphasized the genetic improvement of alfalfa varieties and the development of biotechnology in support of this effort. We have focused our efforts on limitations of alfalfa varieties that have not been adequately resolved through conventional breeding – including yield stability, persistence, and feeding quality. These are consistent with the research priorities of the Ontario Forage Crops Committee and the Field Crops Research & Services Committee.

"We have focused our efforts on limitations of alfalfa varieties that have not been adequately resolved through conventional breeding – including yield stability, persistence, and feeding quality."

1994 Ontario Crop		
Total improved forage	3.6 million acres	> $685 million
Alfalfa & alfalfa based mixtures	2.1 million acres	$405 million

Fig. 1. Total alfalfa acreage and crop value in Ontario.

Research on alfalfa hybrid varieties, pollen control systems, and DNA fingerprinting (see article pg. 7) is underway to improve its yield and persistence potential.



FALFA *biotechnology*

Concurrently, a number of genes are being introduced into alfalfa to improve either its persistence or its feeding value. Improvements in these attributes will lead to reduced feed costs in livestock rations.

Requirements: Commercial Suitability and *In Vitro* Culture

To successfully use biotechnology methods such as gene transformation, the crop must be easily manipulated using *in vitro* culture. Plants which have commercial use must be capable of producing *in vitro* cell cultures from vegetative parts, and regenerating back into plants following the culture cycle.

Following a decade of breeding, a series of populations which combine *in vitro* culture ability, disease resistance, yield, and adaptation have been produced. Not only have we developed plants that grow well in both the laboratory and the field, we have produced plants that work better in tissue culture than the original plants used as the genetic source of this character.

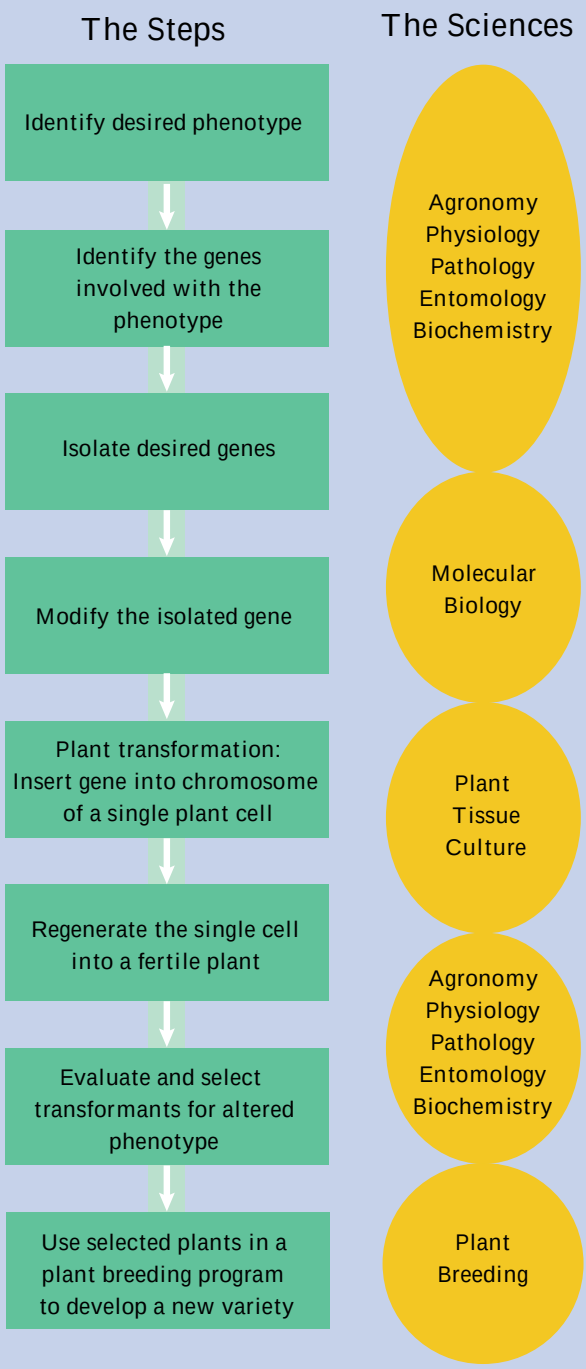
Embryogenic Alfalfa: Evaluation & Plant Performance Improved

Alfalfa populations capable of forming somatic embryos from cell cultures (See sidebar pg. 4) have a number of applications. One of their uses is to provide suitable plant materials for genetic transformations. By transforming plants of commercial utility, we will reduce or possibly eliminate, the amount of conventional breeding required to produce a commercial, genetically engineered variety. We are also better able to evaluate the utility of the added gene in a variety since it will already be in a plant adapted for use in Ontario.

A second application of embryogenic populations is for the production of novel commercial hybrids. Current varieties of perennial forage and turfgrasses are populations derived from many (20 to 100 or more) parents that are increased for three generations. Inbreeding that occurs during the seed increase steps decreases the performance, such that the yields of the seed of commerce (Certified generation) of alfalfa average 7% lower than the first generation (Breeder). By developing hybrid varieties in which the parent plants are vegetatively propagated through tissue culture, this decline in productivity can be avoided and the yield and plant performance maximized.

Field trials of experimental alfalfa hybrid varieties developed

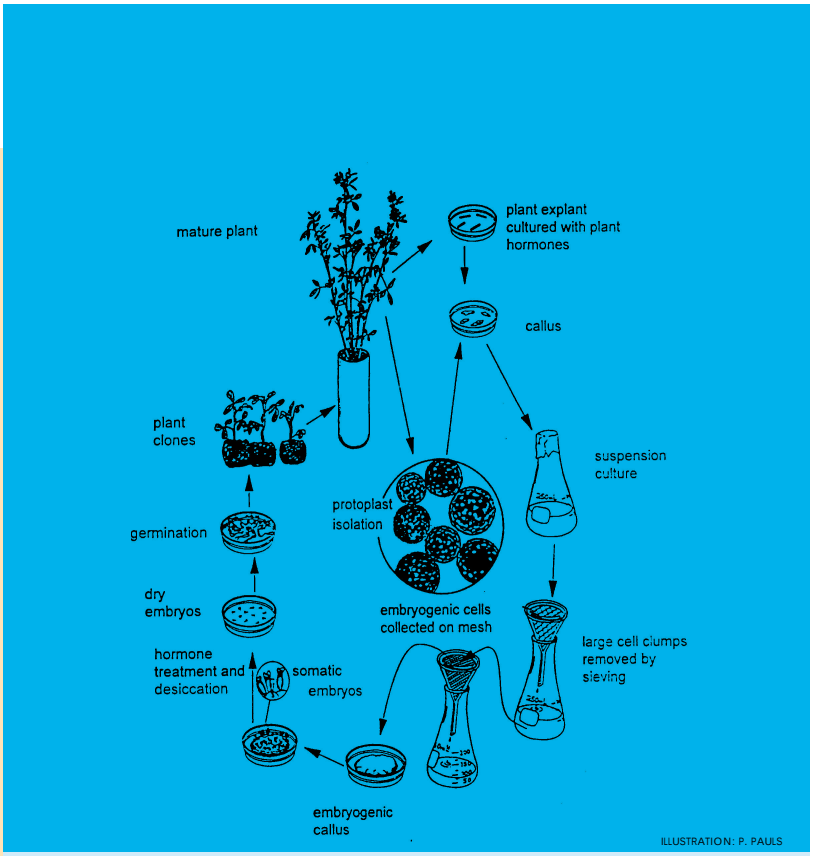
Commercializing Transgenic Plants



Embryogenesis in alfalfa and perennial grasses:

Although alfalfa hybrids have long been recognized as desirable, economical methods of propagating the parent plants have been challenging. One aspect of our research has been the development of in vitro cultures to produce large numbers of somatic embryos, which are genetic copies of the parent plant. We have refined the process to improve the quality and vigour of the embryos produced. For example, we can produce over 1000 embryos from a one cm piece of an alfalfa leaf petiole. We have also developed and patented in Canada, Europe, and the United States a process whereby these somatic embryos can be dried and stored like seed, for extended periods of time. This research has been applied to other plant species including conifers, geraniums, and orchardgrass, and has the potential to alter the present less efficient methods of producing clonal propagules.

Several grasses have been identified which have the capability to form embryos from vegetative plant parts. As was initially the case in alfalfa, the plants that were well suited for tissue culture were not commercially suitable. Beginning in 1992, we initiated a prebreeding program similar to that used for alfalfa in two perennial grasses, orchardgrass and creeping bentgrass, to develop commercial populations suitable to exploit advances through biotechnology. Orchardgrass is a forage grass widely used in mixture with alfalfa while creeping bentgrass is used as a turf grass by the golf industry. With support of OMAFRA and an industrial partner, Speare Seeds, we have improved our ability to manipulate these grasses through in vitro culture.



Plant Tissue Culture and Embryogenesis: Plant cells can be induced to proliferate in a variety of forms including non-differentiated callus and suspension cultures *in vitro*. Single cells like protoplasts express totipotency, i.e. the ability of a single cell to regenerate into a whole plant. Regeneration may occur via various processes including somatic embryogenesis.

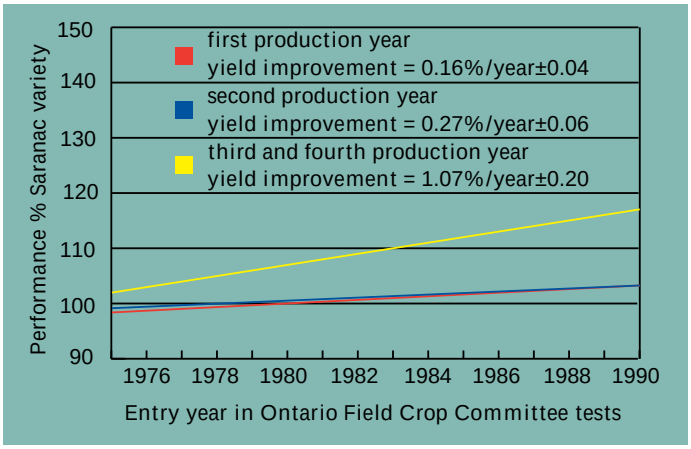
from these populations were initiated in 1995 at several sites in Ontario. Production of experimental orchardgrass varieties will be initiated later this year.

Transformed Plants: Improved Stress Tolerance

One of the major limitations in alfalfa production is winterhardiness. The ability to survive the Ontario winter requires a composite of stress tolerances including freezing, desiccation, ice-encasement, flooding, and disease. Physiological and biochemical research has shown that a common factor among all of these stresses is the production of activated forms of oxygen, or oxygen free radicals. In medicine, these degradative molecules are key components in many physiological disorders, including arthritis, AIDS, stroke and ALS (Lou Gehrig's disease), where they cause degradation of cell structure by attacking lipids, proteins and nucleic acids.

Alfalfa already has a substantial defense system against oxygen including the enzymes superoxide dismutase, ascorbate peroxidase, and glutathione reductase and the antioxidants B-carotene, ascorbic acid (vitamin C), a-tocopherol (vitamin E) and glutathione.

Fig. 2. Genetic yield improvements of alfalfa varieties in Ontario, 1975-1990.

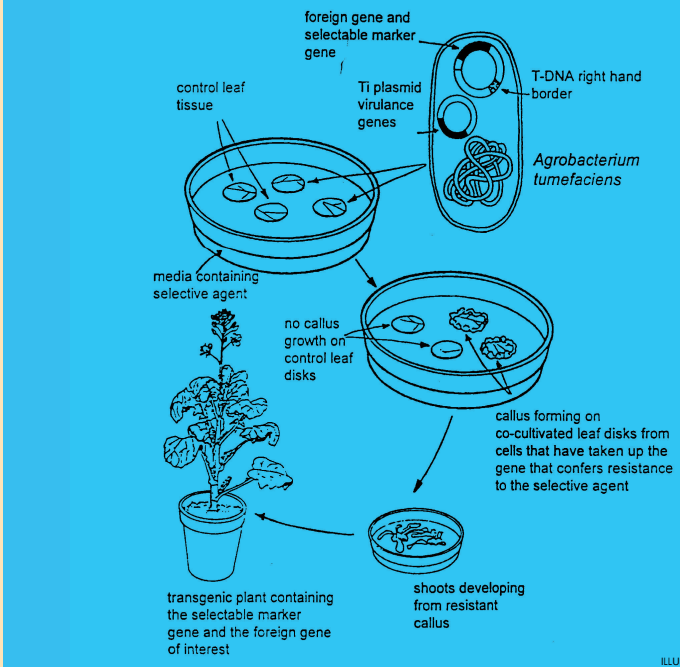


Alfalfa transformation: 8 to12 months

Our alfalfa system uses *Agrobacterium tumefaciens* as a vehicle to insert DNA into cells from petiole explants. The petioles are soaked in the *Agrobacterium* culture, and cultivated together for two days, during which time the T-DNA (transfer DNA) enters the plant cell. Then the petioles are placed in a culture medium containing cloroforan to kill the *Agrobacterium*, 2,4-D to stimulate the formation of somatic embryos, and an antibiotic to select the transformed cells. The transgene is coupled with a gene for resistance to the antibiotic, so that only those cells that had received the T-DNA will grow.

Once the embryos form, they are transferred to a hormone-free medium to allow growth of shoots and roots. Rooted plants are then transferred to the greenhouse, where they are screened using molecular techniques for the presence of the transgene, and using biochemical and physiological assays for the desired phenotype.

Because we use highly embryogenic alfalfa lines, this system can yield up to 100 plants from a single transformation experiment. There is tremendous variability among plants for expression of the transgene, ranging from very low to very high (Fig. 3). We aim for at least 20-40 transgenic plants in order to obtain at least one plant with the desired expression of the transgene. The time required from transformation with *Agrobacterium* to a plant in the greenhouse is about 8-12 months, depending on how fast the seedlings develop roots.



Plant Transformation: A foreign gene, coupled to a selectable marker gene, is cloned into a disarmed Ti plasmid of *Agrobacterium tumefaciens*. The foreign gene and the selectable marker gene are transferred to the plant by cocultivating the bacteria with the explant (leaf disk) and regenerating plants from the cells that express resistance to the selective agent in tissue culture. The foreign gene becomes integrated into the DNA of the plant and is inherited by future generations of plants derived from the original transgenic plant.

Nonetheless, in many growing areas of Ontario, it is insufficient. To bolster this defence system we attempted to increase expression of one of the enzymes, Mn-superoxide dismutase (Mn-SOD). In 1988, when we first tried, we did not have a good agronomic type of alfalfa available to transform so we used the genotype RA3. We also did not have good, cold-responsive promoters, so we used the constitutive promoter from the cauliflower mosaic virus called CaMV35S, that caused Mn-SOD to be expressed in most tissues in the plant. After the first and second winters, these transgenic plants had higher survival and yield than the original non-transgenic variety (Fig. 4).

At this stage, we were faced with a decision. Do we spend a number of years in order to backcross the transgenes from RA3 into commercial varieties, or do we retransform the elite, regenerating genotypes recently developed in the breeding program? We chose the latter approach and have now produced over 100 transgenic plants expressing various forms of SOD in the genotype N4-4-2. These are now being evaluated in the laboratory and will be going into field trials in 1996.

Genetically Engineering Female Plants to Prevent Self-Pollination

Hybrid seed is produced from controlled mating of selected male and female parents. However, alfalfa plants, like most agronomic species, can produce self-pollinated seed during normal flowering. Self-pollination, in which an individual plant is both the male and female parent, leads to reduction in performance due to inbreeding depression. The greater the amount of self-pollination during seed production, the lower the performance of the hybrid variety. To minimize the chance of self-pollination, special measures are necessary during seed production. These measures effect some sort of 'male sterilization', as in corn, for example, where the tassels are removed from the female parent plants to prevent self-fertilization.

Winterhardiness in Wine Grape

Our success at improving the persistence of alfalfa has prompted us to extend this work to a more difficult species, *Vitis vinifera*, in cooperation with an industrial partner, Chateau des Charmes Wines Ltd.. The introduction of high quality wine grapes into Ontario has led to the production of international award winning wines by Ontario wineries, including Chateau des Charmes. With the support of NRC, we have developed a transformation system for grape that is widely applicable to most *vinifera* varieties. The first transgenic grape plants should be going into field trials in spring 1996. A number of candidate genes to improve freezing tolerance are now being inserted into grape. If we are successful in increasing the freezing tolerance by only 2°C, we anticipate that grape production in Ontario might double. Since there are approximately $1 billion worth of grape products sold in Canada, and since we supply only 10% of that with domestic production, there is considerable opportunity to expand grape and wine production in Canada.



One approach in alfalfa is to genetically engineer male sterility in the parent line so that these plants can only be 'female' parents. We have chosen the strategy of eliminating the production of functional pollen in alfalfa through the expression of a 'knockout' gene in the developing pollen grain. This requires the isolation of genes which are expressed only in pollen in order to isolate the promoter regions of these tissue-specific genes. The promoter is an adjacent part of the gene that acts as a switch to turn that gene on, in a specific tissue or in response to certain stimuli such as cold temperature, insect feeding etc..

We have isolated eight such genes in alfalfa which are expressed exclusively or preferentially in pollen. Six of these code for enzymes which are probably involved in germination of the pollen grain, and growth of the pollen tube through the female tissue to the egg. However, two of these genes code for proteins of unknown function and are expressed at an early stage of the development of the microspore. Several of these promoters have been attached to various genes for further studies of their function. One of these studies involves expression in alfalfa of the spike protein of a swine virus, for use in an innovative vaccine project described by Larry Erickson on pg. 47.

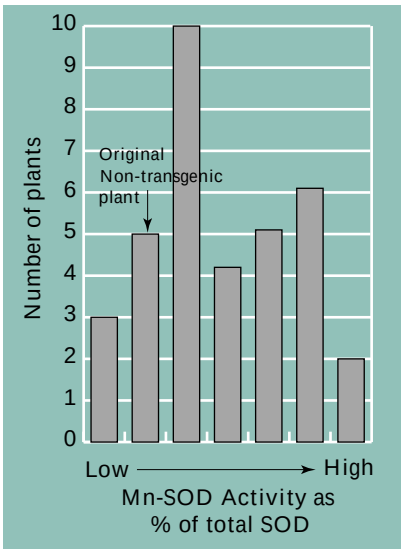


Fig. 3. Variability in Mn-SOD activity among transgenic alfalfa.

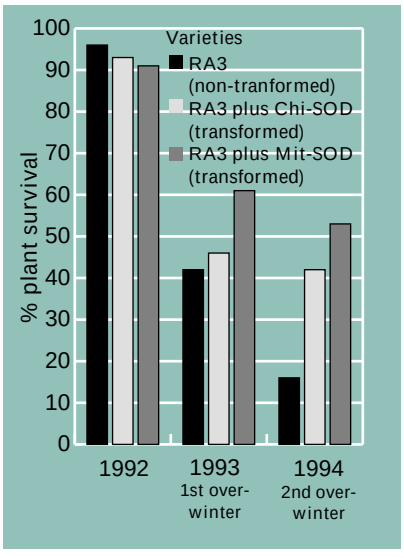
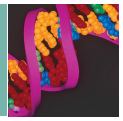


Fig. 4. Over-winter survival of transformed alfalfa varieties.



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MOLECULAR MARKERS:

fast track screening for crop diversity, desireable traits

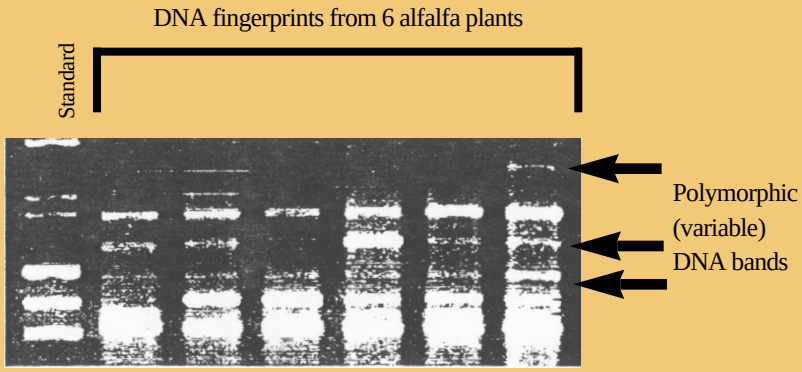


Fig. 1. DNA fingerprints are produced by cutting or amplifying DNA samples from biological organisms to give small DNA fragments that can be separated, according to their size, by moving them with an electric current through a porous slab-shaped gel. The DNA fragment patterns that result from this electrophoresis procedure resemble product bar codes. The DNA bands in this picture were isolated from six different alfalfa plants.

Molecular markers first proved useful when we began DNA fingerprinting to identify the region of the alfalfa genome responsible for the crop's ability to produce somatic embryos in tissue culture. Somatic embryogenesis is a valuable mechanism of propogation, and Steve Bowley, the alfalfa breeder in the Department of Crop Science, is incorporating this trait into a wide variety of alfalfa genotypes with a patented synthetic seed technology developed at Guelph. Researcher Bryan McKersie is also using the somatic embryogenesis trait to produce transgenic alfalfa. (See article page 2).

In our lab, DNA fingerprints were produced from amplifications of specific portions of DNA isolated from the alfalfa plants, using the random amplified polymorphic DNA (RAPD) procedure. This method has several advantages: very little plant DNA is required; no previous information about the composition of the DNA is needed; and the analysis can be completed quickly, usually in less than two days.

Because somatic embryogenesis has a complicated inheritance pattern, identification of molecular markers for the genes that control this trait will allow faster breeding of easily transformed alfalfa varieties, useful for the production of artificial seeds. In molecular marker assisted selection, a plant breeder determines whether a plant will be used for further rounds of selection on the basis of the presence or absence of a molecular marker linked to the trait of interest. The advantages of molecular assisted selection are that: assessments only need a small sample of tissue from the plant, expensive and time-consuming field evaluations are not required and the analysis can be done at a seedling stage.

Researcher Kang Fu of Guelph was successful in identifying a molecular marker for one of the two genes controlling somatic embryogenesis in alfalfa. It was the first demonstration that DNA fingerprinting can be used to analyze the tetraploid form of alfalfa which makes up the majority of the commercial alfalfa germplasm. The technology is therefore, directly applicable to problems facing most commercial alfalfa breeders.

Predicting Success of Hybrid Combinations

In addition to identifying markers for discrete genes, we also found that calculations based on the similarities and differences in the

ocurrence of individual bands in the DNA fingerprints could be used to estimate genetic relationships among alfalfa plants or populations. The usefulness of this to predict which alfalfa plants will do well in hybrid combinations is currently being tested in a joint project with Steve Bowley.

Our initial successes with DNA fingerprinting in alfalfa led to application of the technique to the Lyn Kannenberg's corn breeding program, and to Tom Michaels' bean breeding program. We are also collaborating with researchers from Egypt to define species relationships among plants which are parasitic on crop plants and have hosted visitors from Korea, China and Romania for training in this technology.

In work supported by the Ontario Bean Producers' Marketing Board, we hope to develop selection criteria for breeding disease resistant beans, based on the presence or absence of specific DNA fragments.This technology could replace the cumbersome and variable disease screening procedure that is currently being used. Masters student Yonghe Bai has identified five markers spread over three discrete regions of the bean genome that account for 90% of the variability in bacterial blight resistance in several populations segregating for this trait. Bai and Ph.D. students Bunyamin Ta'aran and Aaron Beattie, are currently testing the utility of the markers for selecting blight resistant material from populations in the bean breeding program at Guelph.

Genetic Distance and Vigour in Corn

The molecular marker technology has also been used by Guelph researcher Daska Stojsin to analyze genetic relationships among corn hybrids used in Ontario, and by PhD student Jon Popi to characterize the lines in the Hierarchical Open-end Population Enrichment (HOPE) breeding program (Vol. 14 No. 4), designed to increased genetic diversity of North American corn germplasm. Jon Popi confirmed that the HOPE germplasm is genetically diverse and distinct from the typical North American corn hybrids. This finding supports current speculation that part of the explanation for the superior performance of hybrids from crosses between HOPE program material and typical North American corn material can be attributed to heterotic vigour introduced into the combination by the diverse genetic background of the HOPE program material.

Our experience with the molecular marker technology shows that it has considerable potential for direct application in a variety of crop breeding programs. We expect to use DNA fingerprinting technology to rapidly screen large numbers of plants in a breeding program at an early stage of development, for genotypes with desirable traits. The technology also gives us information about genetic diversity and structure of the plant breeding populations, which can be used to assess the effectiveness of breeding strategies.

"The [DNA fingerprinting] technology is therefore directly applicable to problems facing most commercial alfalfa breeders."

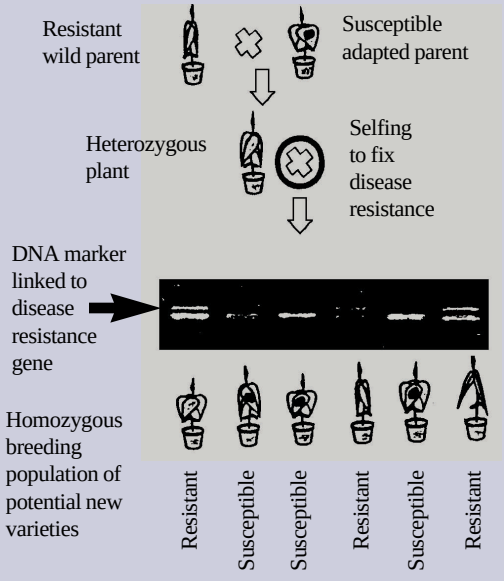
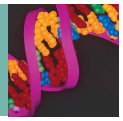


Fig. 2. Associations between individual DNA fragments and genes controlling agronomic traits can be determined by studying the segregation patterns of the DNA fragments, and the genes of interest in plant breeding populations. DNA fragments frequently found in plants that express the trait and are absent from plants that do not have the trait, are called DNA markers. Because DNA markers are not affected by a plant's age or environment and because DNA analyses are quick and can be done at any stage of development (including seedlings), it is sometimes advantageous to select for plants that have the DNA marker, rather than determining whether the plant expresses the trait. This is especially true if the assay for the trait is complicated (for example, a seed quality trait) or is affected by the stage of development or the environment in which the plant is grown. For example, expression of disease resistance is affected by the inoculation procedure, the age of the plant and humidity during the incubation period. The DNA marker indicated by an arrow in this figure can be used to select disease-resistant beans.



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TRAN$FORMED

Barley

The transfer of useful genes or genetic material into crop species from related species has long been employed in improving cultivated crops. The traditional method for barley has been to make hybrids with wild relatives, using embryo rescue techniques. These rare hybrids, when possessing some fertility, can be crossed back to barley with the chance that some chromosome segment of the wild species can be transferred. The resulting barley plant with genetic material from the wild relative, is then tested for useful traits derived from the wild relative. However, since the early 1990s, a biotechnological system of gene transformation has been developed for cereals. In the past year, our lab has achieved transformation of barley.

Transformation has the advantage of being able to add only one or two desired genes to the best current barley cultivars. There are advantages of this technique in barley, compared to most other crops. First, it is a self-pollinating diploid species and haploid phases can be transformed. Second, transgenics in barley are not of concern to the environment because there are no wild relatives or weeds in Canada or North America with which it will hybridize.

Both wide hybridization and transformation have recently been successful in our research programs. From hybrids between barley and its wild Mediterranean relative *Hordeum bulbosum*, we have successfully transferred a segment of a bulbosum chromosome to barley. This segment carries a dominant resistance gene for powdery mildew, a major barley disease in Ontario. The segment can be visualized on the end of barley chromosome No. 2 (Fig.1) by *in situ* hybridization. Barley plants containing this segment have shown resistance to all mildew races so far checked in Ontario and Northern Europe. It appears to be an important new source of resistance and it is being incorporated into our barley breeding program at Guelph. When grown in China, this same barley line also has resistance to the soil borne yellow mosaic virus (Fig. 2), which is the major barley disease in Asia. Lines with this virus resistance have already been released as cultivars in China, where they are well adapted.

New Source of Mildew Resistance

At Guelph, we have been able to incorporate into winter barley a gene conferring resistance to the herbicide *Ignite* (Fig. 3). This was achieved by an unique system of bombarding immature barley pollen (haploid microspores) with very small gold particles coated with DNA containing numerous copies of the gene conferring resistance (Fig. 4). Once the particles are shot into the pollen (Fig.5), the DNA is released and on rare occasions, can be incorporated into the barley chromosomes.

"We have successfully transferred a segment of an *H. bulbosum*" chromosome to barley [which] carries a dominant resistance gene for powdery mildew, a major barley disease in Ontario."

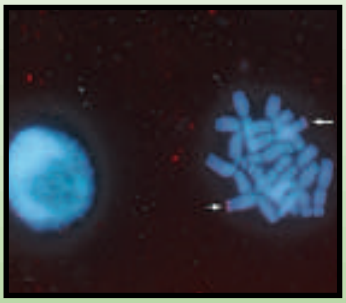


Fig. 1.



Fig. 2.

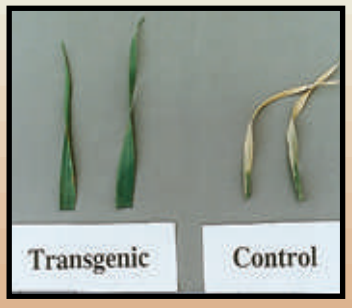


Fig. 3.



Fig. 5.



Fig. 6.

Fig. 1. Chromosomes from a barley plant containing powdery mildew resistance from *H. bulbosum*. *In situ* hybridization with red fluorescing bulbosum DNA reveals the *H. bulbosum* segment on the end of barley chromosome No. 2 (arrows).

Fig. 2. Field grown barley showing the yellow mosaic virus susceptible parent on the left and the resistant progeny (right), transformed with a segment of *H. bulbosum* chromosome as depicted in Fig. 1.

Fig. 3. Barley leaves of a transformed plant (left) and the parent (right), one week after being dipped in the herbicide *Ignite*. (Photo: Q. Yao).

Fig. 5. A viable immature barley pollen with a gold particle (arrow) containing copies of *Ignite* resistance gene, two days after bombardment. (Photo: Q. Yao).

Fig. 6. Results of isolated microspore culture in barley showing germinating embryos about four weeks after culture initiation. Between 2,000 and 3,000 green plants can be regenerated from this 5 cm plate. (Photo: E. Simion).

The immature pollen is then induced to divide and develop into embryos and plants (Fig. 6). During this culture period, a very low level of the herbicide is introduced into the medium so that only those pollen with the herbicide resistance gene incorporated into their chromosomes will survive and grow. This work was initiated in collaboration with Monsanto Co..

Improved Winter Survival and Reduced Fatty Acid Content

Our continuing work will emphasize gene transformation since wide hybridization incorporates a block of genes, many of which could be deleterious to the performance of the barley crop. Of the many traits that could be worked on, we are restricted to those where suitable genes have already been isolated. We have selected two areas for study: the improvement in winter survival of winter barley in Ontario; and reduction of barley seed fatty acid.

When linoleic acid in the barley seed oxidizes, it causes stale beer and poor shelf life. As a result, brewers currently spend millions of dollars on energy to cool the process and for storage. Our research in the improvement of beer storage life is supported by the Brewing and Malting Barley Research Institute. In the area of barley winter hardiness, we are collaborating with W.G. Thompson and Sons Seeds Ltd.. Our major funding for both projects is provided by OMAFRA and NSERC.

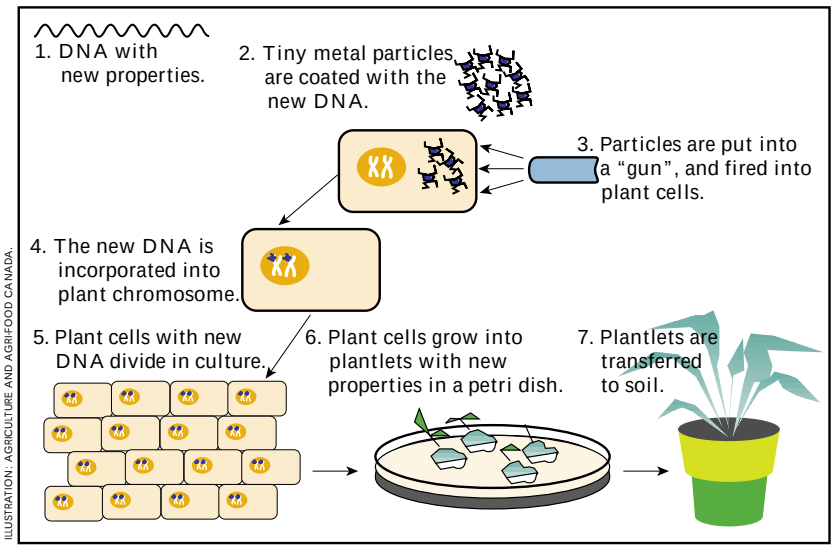


Fig. 4. Particle bombardment for delivery of DNA into plant cells.

Genomic Mapping:

Generating New Genes and Basic Science

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Fig. 1. Petunia varieties used to generate a RFLP (restriction fragment length polymorphism) map of the petunia genome.

Petunias – the name conjures up an image of broad blossomed cheery flowers, staples of the Ontario annual bed, but in their indigenous setting in Central and South America, they grow as woody shrubs with a profusion of tiny flowers running along the stem. The slight stickiness and sharp scent reflect a first-cousin relationship with tobacco, in the solonaceae family. Less obvious is the close evolutionary relationship between petunia and two major horticultural crops, tomato and potato.

For the past year, we have been developing a molecular genetic map of the petunia genome, based on DNA analysis of the varieties shown in Fig. 1 — V23, R51 and Mitchell.

Our aims have little to do with making better petunias. They are, instead, to make new genes available for study or for use in the genetic engineering of other species; to learn what sometimes goes awry when plants are regenerated from tissue culture (a method of increasing popularity for a variety of applications, especially in breeding and propagation); and most fundamentally, to help teach us how nature gives birth to new species.

Defining Linkage Groups, Gene Order and Distances

In petunia there are seven matched pairs of chromosomes, compared to the 23 pairs found in humans, but the total DNA content is about the same. In the production of sex cells a great deal of shuffling goes on between matched chromosomes, but genes which lie close to one another tend to stick together. If we can tell which sets of genes tend to remain together from generation to generation, we can slowly build a map, first defining 'linkage groups', each representing a chromosome, then identifying the order of genes and even the distances between them.

While the genes are often similar in distantly related species, the precise DNA sequence varies even within a species, especially in those spacer regions of DNA that

connect the genes. This variation is the key to constructing an RFLP (restriction fragment length polymorphism) map. The critical word is 'polymorphism', referring to variations in DNA sequence which we can detect by probing the DNA, using previously cloned 'test' genes.

The fragment of DNA carrying a test gene can be visualized as a short band in an Xray film; a variation in sequence which changes the length of the fragment results in the appearance of an additional band. By looking at the banding pattern for a test gene we can determine which copy of the gene was inherited from each parent. If copies of two genes are almost always co-inherited from a hybrid parent, they must be sticking together, and except in rare circumstances, that means they are physically linked, lying near each other on a chromosome.

Using DNA polymorphisms, I test one gene after another, identifying linkage patterns, and slowly building a map of the seven chromosomes. The methods are similar to those used in the human genome project, but our budget is about a fraction of that of the human project.

Cloning Genes Useful in Modifying Other Plants

How does such a map make new genes available, and why do we want them? The petunia map facilitates gene cloning in the same way that the human map has permitted cloning of hundreds of genes associated with human genetic diseases: myotonic dystrophy, cystic fibrosis, and spinal muscular atrophy, just to mention a few cloning triumphs of Canadian medical genetics. Classical mapping, accomplished by looking at co-inheritance of gross physical characteristics, has allowed the mapping of countless traits whose genes remain unidentified. If the gene for a trait of interest maps close to a previously cloned gene on the RFLP map, the available gene can serve as the starting place for a walk along the chromosome to the desired gene. Simple in theory, the process is laborious and expensive. In many cases, however, there is no known alternative.

With petunia, a major aim is to clone genes likely to be useful in modifying other plants. Many petunias are blue, for example, but roses are not. Specifically, blue petunias

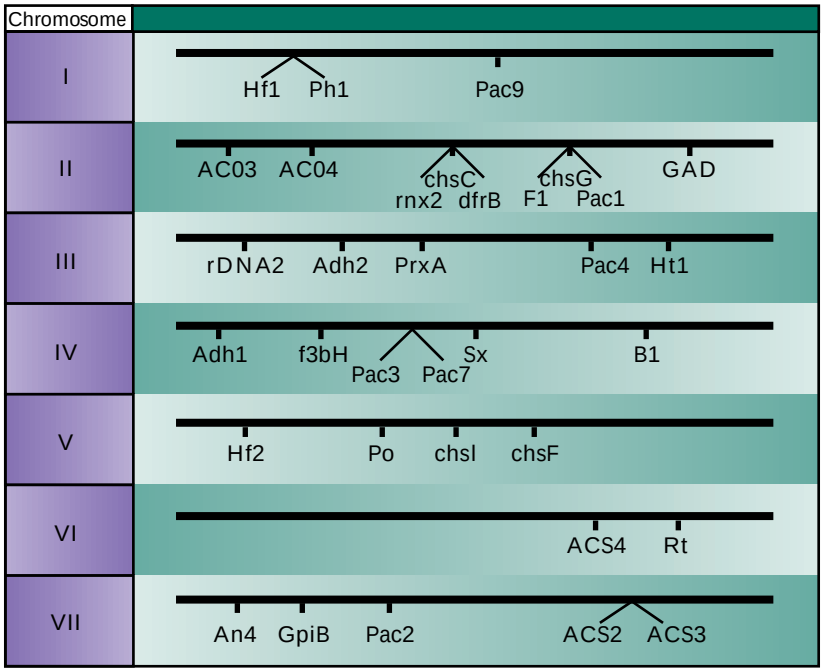


Fig. 2. The working RFLP map, with 34 test genes assigned. In some cases genes map so closely to one another that their order has not yet been determined.

Examples of Genes Mapped

Adh	- alcohol dehydrogenase
Pac	- actin (a structural protein)
*Hf	- modifier of pigmentation
ACO	- aminocyclopropane carboxylate oxidase
*chs	- chalcone synthase
*chi	- chalcone isomerase
*dfr	- dihydroflavonol reductase
*f3ßH	- flavonone hydroxylase
rnx2,Sx	- self incompatibility
*Ht	- flavonal hydroxylase
BI	- blind (closed) flowers
*Po	- pollen colour
*Rt	- glucoside anthocyanin
*An	- anthocyanins
Gpi	- glucose phosphate isomerase
GAD	- glutamate decarboxylase
* Fi	- flavonol quantifier
rDNA	- ribosomal RNA
Prx	- peroxidase
ACS	- aminocyclopropane carboxylate synthase

* genes related to flower colour



have a gene needed to modify pigments so they appear blue, instead of red. Roses lack this gene. There is commercial interest in cloning the 'blue' gene for introduction to roses. Other sorts of genes likely to be of interest are associated with disease resistance, stress tolerance, and even growth habit.

The petunia map may even permit the cloning of desired genes from wild (and often correspondingly more disease – and stress-resistant) varieties of tobacco, tomato and potato. The linkage patterns of genes are surprisingly well conserved among species.

Depending on the particular region involved, chromosome walking can be more like a hike along the Appalachians or a trek in the Himalayas, but unlike the gene order, the sites of troublesome regions tend to be species-specific. Petunia has a well developed genetic system; it is not overendowed with the repetitive DNA elements that make walking difficult, and many genes that can serve as starting points have already been cloned. If a 'chromosomal Mount Everest' is encountered en route to a tobacco gene that confers resistance to some serious fungal pest, for example, we can jump to petunia and walk through the analogous region until we reach the destination side of the block.

Overcoming Plant Tissue Culture Challenges

The map we are also building will also help explain some significant problems associated with tissue-culture based systems of breeding and propagation. The Mitchell line of petunia (Fig. 1) was generated through tissue culture and we have been able to identify specific genetic changes associated with the process. For example, the genes encoding both alcohol dehydrogenases ADH1 and ADH2, have been altered: ADH1 in Mitchell is produced in different tissues; ADH2 is modified structurally and its gene has apparently jumped from one chromosome to another.

While other approaches should enable us to understand the first change in tissue-specific production, molecular analysis based on information in the map should explain the "how", "where" and maybe even the "why" of the second sort of change. While such knowledge has nothing to offer to next year's seed catalogue, it is an important component in developing the best strategies for modern plant breeding and propagation.

Basic Research a Requirement for Progress

The third reason for the map is to satisfy intellectual curiosity – to learn about nature. In countless examples, the usefulness of basic research, not obvious at the start, has a way of revealing itself eventually. While no longer a widely supported rationale for research, the intellectual quest for basic knowledge remains critical to discovery of new technologies and our wise application of them.

"... the intelletual quest for basic knowledge remains critical to discovery of new technologies and our wise application of them".

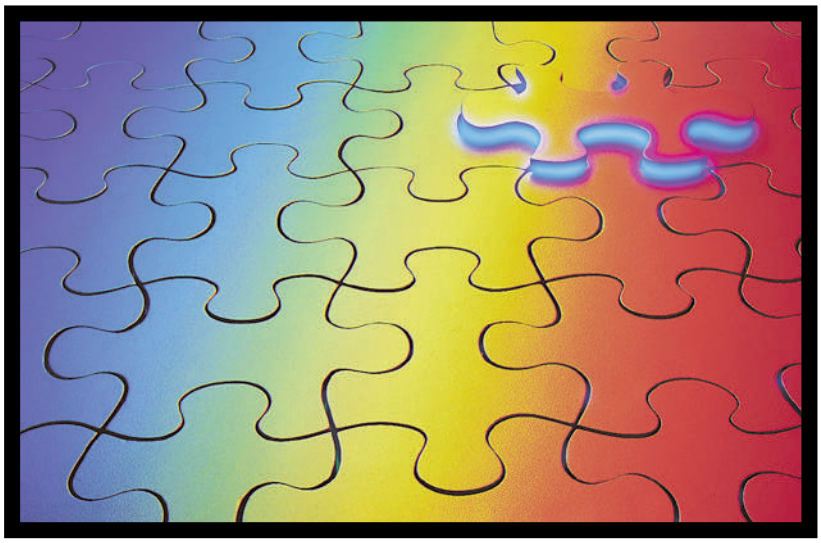


PHOTO: MASTERFILE, MARK TOMALTY

Transformed Cassava:

Improving a Tropical Staple

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There is a long and strong tradition of involvement by the faculty of the University of Guelph in international agriculture. There are especially strong links with research institutions in the tropics, as well as many students from the tropics who have done graduate degrees at Guelph. Since 1991, the Rockefeller Foundation has funded a project in the Department of Crop Science to develop a transformation system for cassava. Cassava is a woody shrub which produces large starchy tubers used extensively as a staple crop in the tropics.

Our initial strategy for gene transfer was to use particle bombardment of axillary meristems (nodal buds) regenerated under conditions to promote multiple shooting. Collaborators in the project included Luiz Vieira of the Agronomy Institute of Parana State, Brazil, who was a visiting scientist and researchers Sautter and Potrykus of the Swiss Federal Institute in Zurich, who developed the microtargeting gun specifically adapted to meristem transformation. The bombardment of many thousands of meristems produced no transgenic plants and the strategy has now changed to cocultivating axillary meristems with *Agrobacterium*, following wounding with bombardment.

Root Deterioration Genes Targeted

Two graduate students from Brazil and Ghana are also engaged in the cloning of cassava microsatellite sequences and genes involved in root deterioration. Microsatellite DNA is a region of a chromosome comprised of short-sequence repeats (eg. ATATATAT) which are highly variable between individuals. For this reason, they are used for genetic fingerprinting of many organisms, including humans, livestock and crop cultivars. This tool is being developed in collaboration with others in the Cassava Biotechnology Network to characterize genetic diversity in wild and domesticated strains of cassava, to facilitate germplasm conservation.

Study of the genes involved in root deterioration is important to the marketing of cassava. The rapid deterioration of cassava roots following harvesting has impeded marketing of the tubers and prevented economic return to farmers who might grow more than their needs to supplement their income. Work continues by graduate students on our team who are supported by their home governments and Canadian foreign aid agencies.

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MICROPROPAGATION:

Mass production of disease-free horticultural crops.

The North American market for herbs is estimated at $500 million annually. New micropropagation technology for mass production of horticulturally important crops has the potential to overcome many of the limitations of conventional propagation methods.



Axillary bud culture has been effectively used in our lab for mass propagation of herbs such as french tarragon, pictured here.

"Artificial seed technology: reduced production costs, handling, storage and shipping convenience, and disease-free plants."

In our lab, we have effectively used the technology of axillary bud break and shoot formation for mass propagation of herbs such as french tarragon (*Artemisia dracunculus*, a culinary herb), and the somatic embryogenesis route of micropropagation for lemon-scented geraniums (*Pelargonium* sp. used in potporri, perfumes and as an insect repellant).

Overcoming Seasonal Varieties and Increasing Plantlet Numbers

The major limitations faced by the herb industry in conventional propagation of french tarragon are the production of a limited number of propagules through cutting propagation, and the season-dependent stock plant suitability for the production of cuttings. The axillary bud culture system developed for french tarragon enables us to overcome the seasonal variations in cutting propagation and to dramatically increase the number of plantlets which are potentially possible from each stock plant.

The somatic embryogenesis system developed for lemon-scented geraniums permits production of embryos on small pieces of plant tissue under sterile *in vitro* culture conditions. These somatic embryos, which are similar to zygotic embryos present in natural seeds, can be dried and enclosed in a natural gel to obtain 'artificial seeds'. Commercial exploitation of the artificial seed technology will aid in the rapid mass multiplication of plants, reduced production costs, convenience of handling, storage and shipping, and will minimize import-export restrictions due to the disease-free nature of the *in vitro*-derived plants.

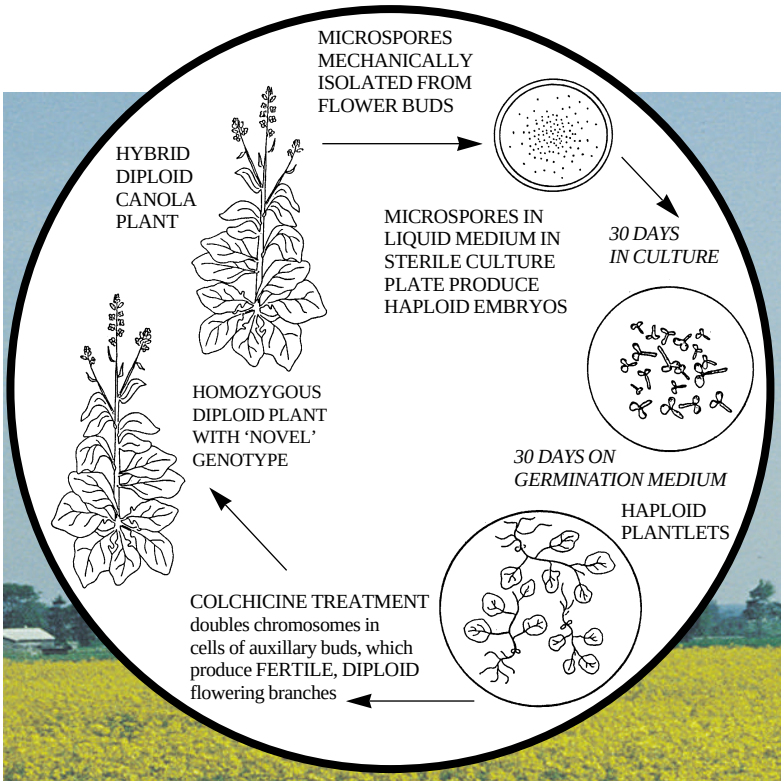
Further application of both these techniques in mass propagation of medicinal plants (such as feverfew, *Tanacetum parthenium*) is currently being investigated.

Acknowledgements: This research program is jointly funded by Agriculture and Agri-Food Canada, NSERC and Richter's Herbs of Goodwood, Ontario.

Fig. 1. Haploid technology - steps in the rapid production of large numbers of homozygous plants from immature pollen grains (microspores).

DOUBLED

accelerates ca



One of the most exciting biotechnical advances in crop plant breeding in the last decade has been the ability to produce haploid plants, particularly in canola. Haploid technology is an extremely valuable plant culture method, providing plant breeders with the capability of rapidly producing large numbers of homozygous plants from immature pollen grains, called microspores (Fig. 1).

HAPLOID TECHNOLOGY

nola breeding



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In this new technique for canola (Brassica napus) breeding developed in our lab, microspores are mechanically extracted from the anther and induced to develop directly into haploid embryos rather than pollen grains, when cultured in an enriched liquid medium. Within two months, embryos germinate and develop into haploid plants, which are subsequently treated with colchicine to induce chromosome doubling. This results in doubled haploid plants with fully restored fertility.

Benefits for Crop Breeding

High frequency embryogenesis from cultured pollen grains of *B. napus* has led to obvious applications in genetic improvement of canola. The direct benefits to researchers in using this breeding method are:

benefit #1 Doubled haploid plants are homozygous and therefore are true breeding.

benefit #2 Pollen taken from hybrids produce haploid plants that represent the recombinant genetic array.

benefit #3 The rapid development of microspores into homozygous diploid plants within eight months dramatically accelerates inbred or cultivar development. By comparison, conventional breeding methods require a minimum of five years.

benefit #4 Direct development of microspores into embryos eliminates incidence of genetic anomalies, in contrast to other single cell culture systems with an intermediate callus stage, where genetic alterations can occur.

benefit #5 Microspore-derived haploid embryos provide ideal targets for genetic engineering, since insertion of foreign DNA into the haploid genome allows homozygous expression after colchicine doubling.

Efficient and rapid production of haploid embryos from immature pollen grains also provides an opportunity for plant breeders to combine *in vitro* mutagenesis with selection for novel and desireable genotypes. Large numbers of haploid embryos and plants can be manipulated at one time with little cost and labour.

Herbicide Resistance and Other Useful Genotypes

Novel canola genotypes can be created in the laboratory by inducing point mutations in cultured microspores, before embryos begin to develop. Mutations induced in haploids in this manner are readily expressed, whether dominant or recessive. This simplifies the selection process for haploid embryos or plants carrying mutations that result in beneficial agronomic or quality traits in the plant or seed oil. Haploid cultures are easily mutagenized *in vitro* during any stage of embryo initiation or development. Used together with *in vitro* selection of canola microspores, genotypes have been identified that carry beneficial mutations.
Ideally altered traits should be readily selectable.

Initial research involving directed gene modifications by mutagenesis involved selection for herbicide resistance. Several canola cultivars have been developed that are resistant to families of herbicides (chlorosulfuron, Glean®; glyphosate, Roundup®; imidazolinones Pursuit®) using this method.

Improving the Fatty Acid Profile

Canola oil has gained the reputation as being one of the healthiest vegetable oils for human consumption because it has the lowest level of saturated fatty acids at 6%, a desirable high level of both mono-unsaturated fatty acid (oleic acid 60%) and omega-3 fatty acid (10%) and a reasonable amount of essential fatty acids (20%). Recent nutritional studies indicate that oleic acid reduces the level of harmful low density lipoprotein cholesterol in humans. From a human health perspective, canola oil could further benefit from an increased level of mono-unsaturated oleic fatty acid content and reduced level of saturated fatty acids.

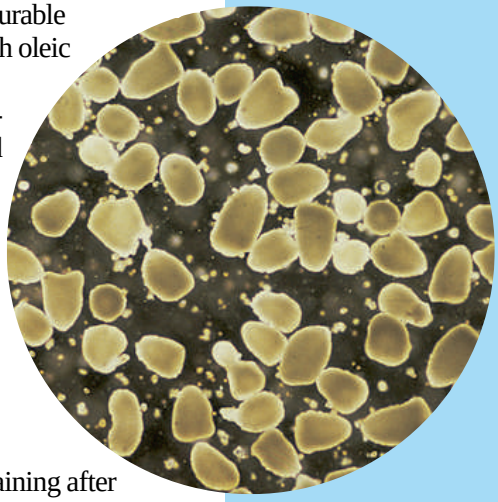
In vitro microspore derived embryos show an oil accumulation process that is similar to in vivo seed embryos. Elevation of oleic acid levels from 60 to 85% with accompanying decrease in linolenic fatty acid from 10% to 3%, have been reported using haploid technology and mutagenesis. Plants carrying this mutation produce oil favourable both to human health with high oleic acid and to industry, offering greater shelf life and oil stability with lowered linolenic acid content.
Doubled haploid lines have now been identified with a reduced saturated fat content of 3.6%.

Reducing Glucosinolates for Livestock Feed

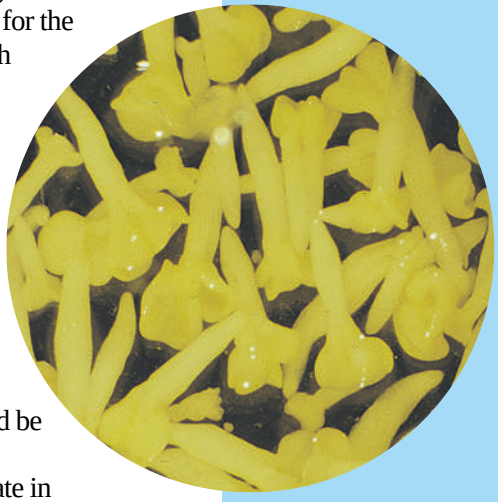
Seed meal is the product remaining after oil has been extracted from the seed. Members of the genus *Brassica* characteristically accumulate undesirable compounds such as glucosinolates in seed and vegetative tissues, which are responsible for the pungent aroma associated with cabbage, other cole crops and mustards. Classical breeding methods have eliminated much of these compounds from the seed. However, since canola meal is an excellent source of concentrated protein for livestock feed, a further reduction in the total glucosinolates in canola would be worthwhile.

Glucosinolates accumulate in a similar manner in both seed embryos and cultured embryos. I*n vitro* mutagenesis combined with selection through testing individual haploid embryos using a non-destructive technique, has identified haploid embryos that have a further reduction of glucosinolate levels in canola breeding lines, and a substantial reduction in a related oilseed species, *Brassica carinata*.

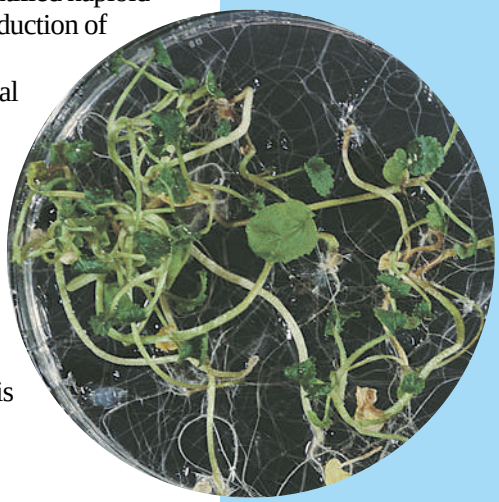
The doubled haploid canola breeding method was developed at the University of Guelph, in the Crop Science Department with financial support from OMAFRA and NSERC, and is now used by many commercial and public plant breeders worldwide.



10 day old canola microspore embryo in culture.



28 day old canola microspore embryo in culture.



Haploid embryos germinated into haploid plants *in vitro*, ready to be transfered to soil and doubled.

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Help for horticultural growers:

Disease diagnosis quick and sure

Ontario crops are attacked by a wide range of plant pathogens. Some are easily identified, based on pathogen structures on the plant or by symptoms caused by infection. Others require specific diagnostic tests. Traditionally, this has meant isolating and growing the microbe in culture and then examining its morphology and physiology – a time-consuming and expensive process which doesn't work for all pathogens. In recent years, biotechnology has provided scientists with new identification techniques.

My research has concentrated on developing assays to detect specific DNA sequences of several important pathogens of Ontario crops, using the polymerase chain reaction (PCR), (see sidebar on page 20).

These include the bacterial pathogens causing aster yellows disease of vegetables such as carrots, celery and lettuce; leaf scorch disease of landscape trees such as elm and oak; and angular leaf spot of strawberries. Assays have also been developed for fungal plant pathogens causing blackleg disease of canola and crucifers; necrotic ring spot and take-all patch of turfgrass; and gummy stem blight of melons. These assays have been used to monitor the pathogens directly in diseased plant tissue, infected seed, infected transplants and infected insects which can transmit the pathogen while feeding. The assays are rapid, highly specific and highly sensitive.

Non-Chemical Disease Control

The benefits of a highly specific assay are evident, for example, in diagnosis of the blackleg pathogen. Two types of this pathogen attack crucifers. One type is weakly virulent and causes only minor damage; however, it is identical in appearance to the highly virulent type which causes stem cankers and major crop losses. Like many pathogens, this fungus can be transmitted by infected seed, which spreads the disease into new areas and initiates major disease epidemics. We developed a DNA assay to differentiate between the two types, and have used the assay to monitor seed for infection by the highly virulent type. By more accurately monitoring seed for pathogens, we can improve our ability to provide growers with pathogen free seed – a non- chemical means of controlling plant diseases.

The high sensitivity of a DNA assay enables us to detect pathogens before they actually cause disease. For example, transmission of the angular leaf spot bacterium on strawberry transplants is a serious concern. In order to prevent the spread of this pathogen, transplants are monitored for disease before

they are sold; however, this disease continues to be a significant problem for Ontario strawberry growers. We were able to use a molecular assay to screen apparently healthy transplants to detect low populations of these bacteria that were present on the plant surface, before disease symptoms appeared. By detecting infected but healthy-looking transplants, we can improve our ability to provide Ontario strawberry growers with pathogen-free transplants.

Info for Integrated Pest Management

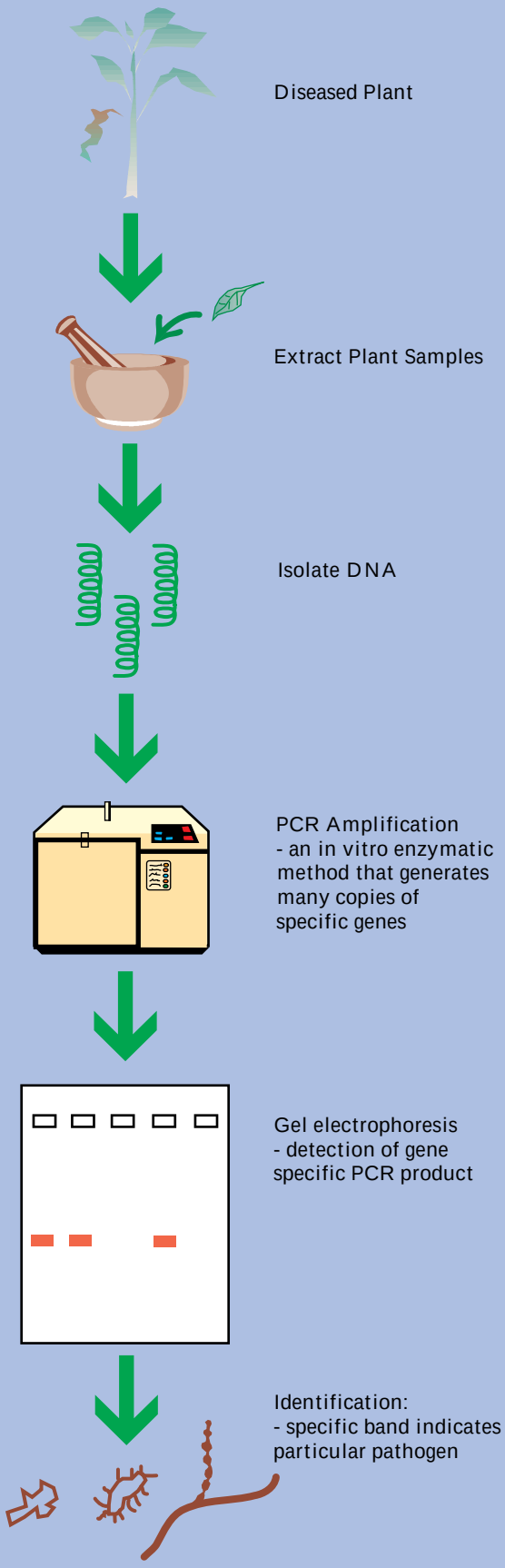
Another advantage of a DNA assay is that it can detect organisms that are difficult or impossible to grow in pure culture. An example is the aster yellows pathogen, transmitted from plant to plant by leafhopper insects. The bacterium cannot be cultured and is in very low concentrations in both the leafhopper and infected plant. The assay that we developed is sufficiently sensitive that it can detect the pathogen in individual leafhoppers, and there is no need to culture the microbe. This rapid and simple assay could help the IPM program identify appropriate times to recommend control of leafhoppers to prevent the spread of the disease.

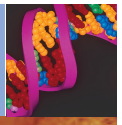
These are just a few examples of biotechnology's value in controlling plant diseases in Ontario. Research is ensuring that these new developments are applied to our crops for the benefit of consumers and the agri-food sector.



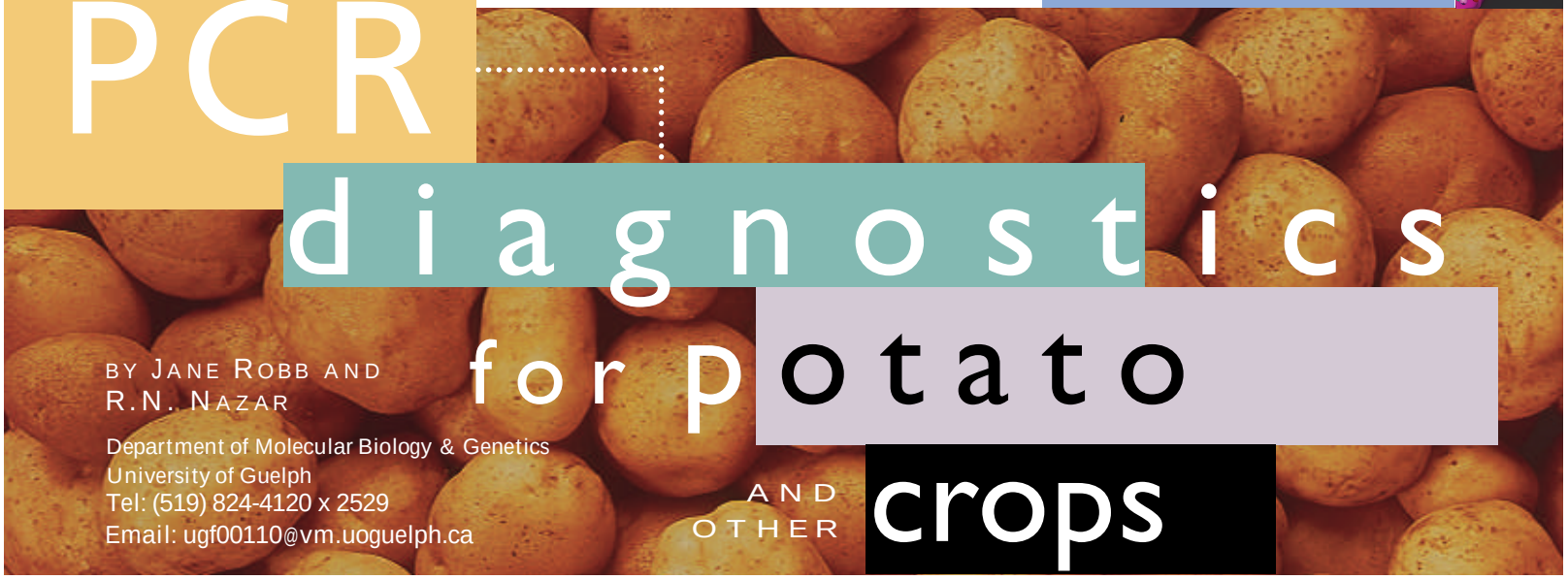
DNA assays have been developed for economically important pathogens of Ontario crops. These include bacterial pathogens causing leaf scorch disease of landscape trees (left) and blackleg disease of canola and crucifers (right).

Detection of specific DNA sequences of pathogens using Polymerase Chain Reaction (PCR)





PCR diagnostics for potato and other crops



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PHOTO: FOODLAND ONTARIO

Verticillium wilt and early dying syndrome are serious diseases wherever potatoes are grown. Constant and rapid monitoring of pathogen levels are important since potato cultivars differ widely in levels of resistance to various _Verticillium_ species such as _V. dahliae, V. albo-atrum_ and _V. tricorpus_. Climate or cultivar changes can lead to critical shifts in the occurrence or predominance of species. These factors emphasize the need for a quick and accurate method of pathogen identification and quantification for producers or inspectors, since control options differ depending on pathogen type.

Identification, Quantification Necessary for Control

To-date, the main method of detection has been field inspection, even though *Verticillium* wilt can have the same symptoms as excess chemical top-killer, drought stress or mature plant senescence. Current laboratory methods which are somewhat more accurate usually require pathogen isolation and identification. The process is time consuming (up to 7 weeks) and labour intensive. Results are at best semi-quantitative and most tests fail to distinguish possible species adequately.

In recent years, we have developed polymerase chain reaction (PCR)-based diagnostics to monitor *Verticillium* species in potato and other crops. PCR is a method for amplifying a segment of DNA that is unique to the particular organism. Such assays are fast, very sensitive, and can be applied quantitatively. Small differences in the DNA sequence of the intragenic transcribed regions of the ribosomal genes have been used to design primer sets capable of distinguishing between species of Verticilium. We have also used genetic engineering to make corresponding internal control DNAs which are also amplified by the specific

> "An important component of this research has been adaptation for the large scale use required by government and commercial testing laboratories."

primers and can be added in fixed amounts to each reaction to serve as a standard for quantification. Methods have been developed for extracting sample DNAs from both plants and soils.

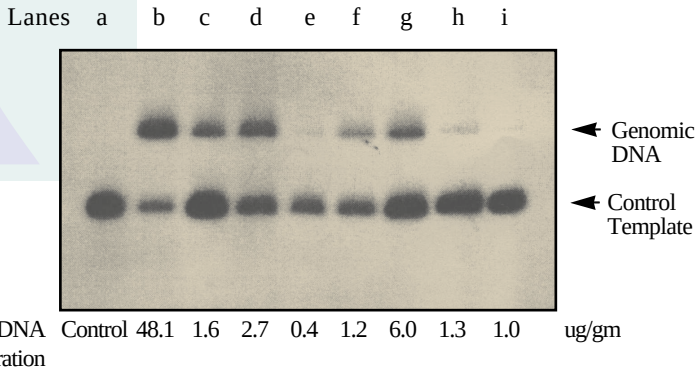


Fig. 1. Quantification of *V. albo-atrum* DNA in stems of severely diseased potatoes. Plant extracts were diluted 50-(lanes d-f), 200-(lanes b,c) or 500-(lanes g-i) fold, depending on the levels of inhibitors. Control DNA was added and the samples amplified with *V. albo-atrum* specific primers. Lane a contains only control DNA. The actual amounts of fungal DNA (g/gm plant tissue) are recorded beneath each sample lane.

Scale-Up for Commercial Use

An important component of this research has been adaptation for the large scale use required by government and commercial testing laboratories. We have worked closely with OMAFRA and Agriculture and Agri-Food Canada in Ontario and Prince Edward Island, two major potato growing regions. Successful development of large scale PCR diagnostics must be based on the following principles:
(1) The assay must be very direct to minimize labour and cost requirements, and,
(2) it must also yield reproducibly quantifiable results over a broad range of pathogen levels and potential environments.

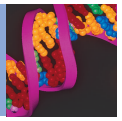
Important features of the methods we have developed at the University of Guelph which facilitate large scale application include:

Feature #1	A single step extraction procedure, which requires no prior purification steps other than grinding in liquid nitrogen;
Feature #2	A simple modification of the extraction procedure which minimizes nucleic acid degradation and losses due to absorption in soil samples;
Feature #3	A simple method to minimize or eliminate the effects of inhibitory substances without additional purification;
Feature #4	Use of internal control DNAs which compensate for differences in efficiency and inhibition with alternate samples;
Feature #5	Development of a two step PCR amplification as a means to increase sensitivity.

These methods can be up-scaled while maintaining competitive cost. They also are applicable to a broad range of agricultural pests and other organisms, and will increase our ability to monitor the health of our environment.



PHOTO: MASTERFILE/G.BISS



Engineering Pathogen-Derived Resistance to Potato Leafroll Virus



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Ontario is the largest producer of potato chips in Canada. The average value of the potato crop in the province is estimated at $40 million per year, but it is highly susceptible to viral, fungal and bacterial diseases. Vegetative propagation is responsible for the transfer of diseases from one clonal generation to the next. The amount of chemical pesticides applied to the potato crop exceeds that of other food crops, making potato production costly and environmentally unfriendly. The Food and Inspection Branch of the Agriculture and Agri-food Canada (AAFC) runs a comprehensive and costly potato seed certification program to control the spread of viral diseases.

Development of disease resistant varieties has been and continues to be a major goal of plant breeding programs. Many problems are encountered, however, when introducing natural disease resistance into potatoes through conventional breeding, such as complexity of genetic inheritance due to polyploidy; difficulty of crossing between sources of resistance which are mostly diploid (2x) and cultivated tetraploid (4x) varieties; loss of agronomically important characteristics due to wide crossing; and, emergence of new strains of pathogens.

Potato leafroll virus (PLRV) is the most serious virus affecting the potato crop throughout the world (Fig. 1). It is transmitted from plant to plant by aphids, the most important being the green peach aphid. PLRV can result in considerable yield losses and is a major cause for early rejection of potato seed production fields in Canada.

Interfering with Replication of the Virus

Genetic engineering is now being used to enhance crop breeding programs. Using this technology, a specific gene is selectively introduced into a cultivar which is suitable for

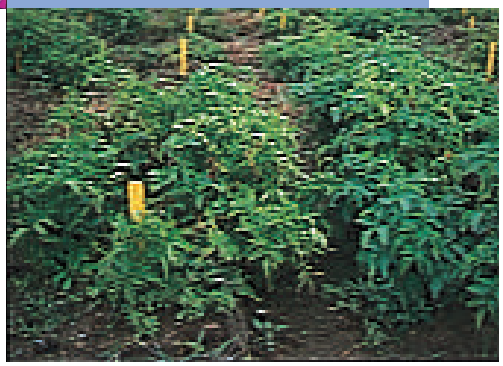


Fig. 1. Potato leafroll virus infected plants (left) and transgenic resistant plants (right).

production, but lacks a trait such as resistance to a disease.

Plant viruses are mostly composed of single stranded RNA encapsulated in a protein coat. It has been observed that the expression of a viral DNA sequence in plants interferes with the replication of the virus, which is essential to establish infection and which controls development of the disease. The molecular basis of this phenomenon is not well understood.

We have transferred the coat protein (CP) gene derived from the pathogen PLRV into two commercially grown potato chipping cultivars, Snowden and FL1607, to develop PLRV resistance. The CP gene was previously isolated by colleagues at the AAFC Vancouver Research Centre. *Agrobacterium tumefaciens* was used to transfer the CP gene along with a kanamycin resistance gene as selection marker into the potato genome.

Potato leaf and stem tissues were inoculated with *Agrobacterium* and cultured *in vitro*. Transformed cells were selected in the presence of the antibiotic kanamycin, and regenerated using tissue culture techniques. The regenerated plantlets were transferred to the greenhouse . A large number of transgenic lines were developed and assessed for incorporation and expression of the CP gene through a range of molecular and biochemical tests.

Field Assessment of PLRV Resistance

Eighty-four transgenic lines were evaluated for stability and effectiveness of the engineered resistance to PLRV and their agronomic performance. The transgenic lines were multiplied by nodal cuttings in the greenhouse and seedlings were planted in the field at Cambridge and Simcoe in replicated trials. Plants were inoculated using infected green peach aphids and tested periodically for accumulation of PLRV using ELISA. Transgenic plants varied for the level of resistance; some of the transgenic lines showed 100% immunity to PLRV (Fig. 2).

> . . . successful conclusion of this project would contribute a minimum of $5 million annually to Ontario potato growers through improved yields and quality of the crop.

We are now conducting detailed molecular analyses to understand the genetic basis of pathogen-derived PLRV resistance. Transgenic lines showing resistance to PLRV and having required agronomic traits would be selected for release to growers. We estimate that the successful conclusion of this project would contribute a minimum of $5 million annually to Ontario potato growers through improved yields and quality of the crop. A PLRV resistant potato cultivar would be particularly useful in developing countries which can not afford costly seed potato production and inspection systems.

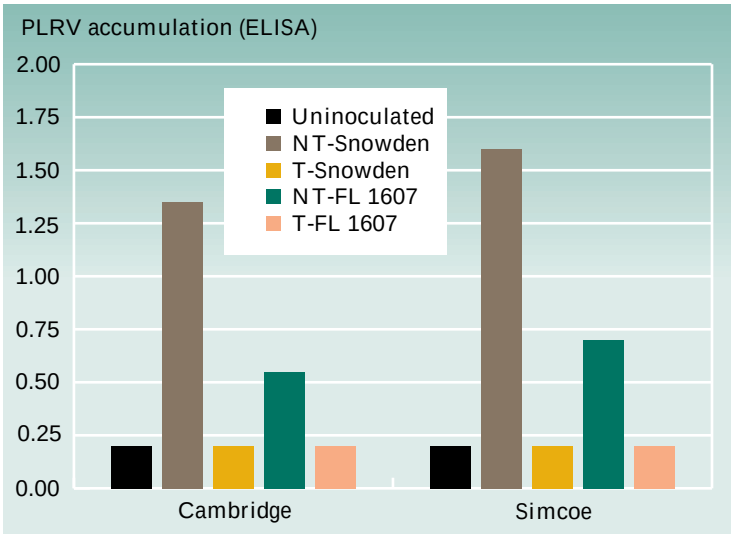


Fig. 2. Potato leafroll virus accumulation in transgenic (T) and nontransgenic (NT) plants, as determined by ELISA.

Acknowledgement: This project is partially supported by funds from AAFC and OMAFRA to S.T. Ali-Khan and a scholarship from the Ministry of Agriculture, Iran to J. Mozafari.

Managing Aggressive *Trichoderma* in Mushrooms

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Green moulds caused by *Trichoderma* species were once recognized as indicators of poor compost quality and were of minor significance in the cultivation of commercial mushrooms, *Agaricus bisporus*. But since 1992 in Ontario, *Trichoderma harzianum* has become a destructive disease. Mushroom composts infected with *T. harzianum* do not generally yield any mushrooms. Frequently, red pepper mites, *Pygmephorus* sp., associated with the infection swarm onto the mushrooms, feeding on the pathogen and causing product loss.

Aggressive Green Mold Identified Prior to Signs of Disease

The Trichoderma research program aims to develop a rapid procedure for identification of the aggressive form of T. harzianum, and identify factors that influence its growth, survival and spread.

A faster diagnostic test than the current two to three weeks would tell the grower whether or not the farm is at risk for a green mould problem, and permit early intervention to control spread of Trichoderma.

With a DNA fragment amplified using the polymeras chain reaction (PCR), we have verified that the test we have developed is highly specific for the aggressive strain of T. harzianum, with results available in two to three days. Every aggressive strain tested to date has yielded a positive result in a variable series of studies on test specificity with purified axenic cultures. No other Trichoderma isolates, including other strains within the harzanium species, have tested positive.

Aggressive green mould has been identified with this test in compost or casing samples, even before the appearance of any disease symptoms.

Factors Influencing Disease Manifestation

Research at the new mushroom research facility at HRIO has demonstrated that the most vulnerable period for disease infection is before the mushroom mycelium becomes established in the compost. The later the spawned compost is inoculated with T. harzianum, the less severe the damage to compost and casing layer. When casing was inoculated with T. harzianum, disease developed 25% of the time and more slowly than in compost.

Compost inoculated with green mould organism and supplemented with a commercial compost supplement, commercially treated with a unique mould inhibitor, produced significantly smaller T. harzianum colonies than composts with or without the other compost supplements. Application of fungicides chlorothalonil, benomyl and thiabendiazole to the surface of spawned compost did not prevent the growth of T. harzianum to the casing material. Examination of diseased areas after post-crop heat treatment has demonstrated that the pathogen can survive temperatures of 65°C, held for up to 20 hours.

To prevent damage caused by T. harzianum, growers must first know if the green mould observed is actually the aggressive one, and then focus their attention to prevent compost infection prior to casing. The preventative measures include: rigorous sanitation and hygiene programs, restrictions of movement of personnel, selection of appropriate supplements and finally, pest management.

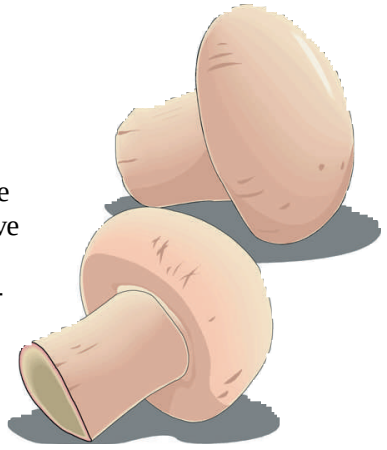




PHOTO: MASTERFILE, PETER GRIFFITHS

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Hypovirulent

STRAINS OF PLANT PATHOGENS:

A NEW PEST MANAGEMENT TOOL

Case Study: dollar spot disease

Dollar spot of turf is a widespread and destructive fungal disease of many turfgrass species, including those used in golf course putting-greens, closely-mown fairways, bowling greens, and home lawns. Disease symptoms include circular, straw-coloured patches (Fig. 1) that often look a dollar coin, hence the name of the disease. In severe cases, the patches grow together to form large areas of diseased turf. This disease is currently managed by repeated applications of fungicides, but it is expensive and being discouraged by public concerns regarding the safety of pesticide use in turf. Dollar spot is one of the largest target diseases for applications of fungicides in North America. Development of alternative methods for controlling this disease will result in substantial reduction of pesticide use in urban environments.

Naturally Occuring Fungal Viruses

Researchers at the University of Guelph have been developing a biological control method using naturally occurring fungal viruses. These viruses are found in some strains of dollar spot, but most do not reduce the ability of the strain to cause disease. After screening numerous strains of dollar spot, we identified three containing fungal viruses that reduced or prevented the pathogen from causing disease (Fig. 2). Such strains are called hypovirulent. Hypovirulent strains of dollar spot can be used as biological control agents because, when applied to virulent strains causing disease in turf, the virulent strains are infected by the fungal virus and become hypovirulent.

From 1993-95, the most promising hypovirulent strains were grown in the laboratory and applied to turfgrass infected with dollar spot at the Cambridge and Guelph Research Stations. These field experiments were designed to determine the best formulation of hypovirulent strains, the influence of timing and frequency of applications on disease control, and the duration of disease control after applications.

The best formulation was a foliar spray of the hypovirulent strain that suppressed dollar spot by up to 80% – a level of control as effective as applications of fungicide. Disease suppression caused by the hypovirulent strain

varied among the treatment and rating dates, but the largest suppressions of disease in these trials ranged from 55-80%. One application of the hypovirulent strain was as effective as multiple applications in suppressing dollar spot.

Vegetative Compatability Important

One of the challenges in using hypovirulent strains for biological control is to determine if the hypovirulent isolate can affect all strains of dollar spot. The viruses found in hypovirulent strains are only transmitted to healthy strains when cells of the two strains naturally fuse, a process called anastomosis. Strains that fuse and transmit hypovirulence are called vegetatively compatible and those that cannot fuse are vegetatively incompatible. Hypovirulence does not usually spread between incompatible strains.

Little information is available on the presence of vegetative incompatibility among strains of dollar spot, so emphasis in 1995-96 has been to evaluate the extent of vegetative incompatibility in dollar spot in Ontario. These results would determine if hypovirulent strains were effective against dollar spot at all locations, or only in selected locations where virulent strains are vegetatively compatible with the hypovirulent strains. Preliminary results indicate that incompatibility is present among some strains of dollar spot within Ontario, but at fairly low levels. We are currently evaluating the ability of our most promising hypovirulent strains to infect these incompatible strains, to determine if this is a significant factor restricting the use of hypovirulence as a biological control strategy.

In summary, the use of hypovirulent strains of plant pathogens for controlling plant diseases represents a new technology for pest management. Our results have clearly established that hypovirulent strains of dollar spot are as effective as applications of fungicide for the control of this disease. The best formulation of the hypovirulent strain suppressed disease as well as a currently recommended fungicide during two years of field trials, and single applications of the hypovirulent strain were as effective as multiple applications of fungicide. In addition, the efficacy of the hypovirulent strain persisted into the next growing season. Our results indicate that the hypovirulent strain is fusing with naturally occurring virulent strains of the pathogen and transmitting the virus-like agent to the virulent strains. Recipient virulent strains then become hypovirulent and have a reduced ability to incite disease or survive, or both.

> "Our results have clearly established that hypovirulent strains of dollar spot can be as effective as applications of fungicide for the control of this disease."

Safety, Fate, Persistance to be Determined

Identification of an effective biological control agent for dollar spot of turf is an important first step. However, continued field evaluations in different locations and environments, and an environmental assessment of the safety, fate and persistence of these agents will be required before such a product can be registered as a pest control product in Canada. In addition to dollar spot, we are currently examining the potential of hypovirulent strains of other important pathogens for biological control of plant diseases.

Our research from 1993-95 was sponsored by the Ontario Turfgrass Research Institute (OTRF), OMAFRA, and NSERC.

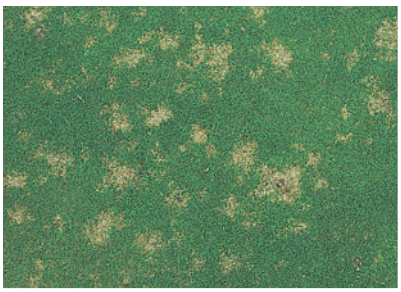


Fig. 1. Symptoms of dollar spot in turf. It is one of the largest target diseases for application of fungicides in North America. Guelph researchers have been developing alternative, non - chemical methods of control.

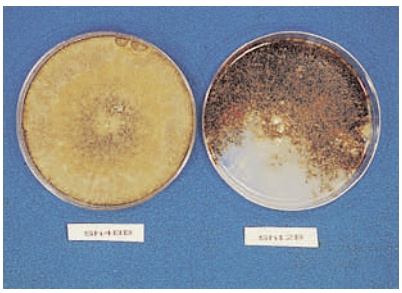


Fig. 2. Virulent (left) and hypovirulent (right) strains of dollar spot. Hypovirulent strains show promise as biological control agents of disease.



Integrating BIOTECHNOLOGIES into genetic improvement of livestock

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Modern livestock improvement makes use of increasingly complex technologies. Extensive recording schemes now capture information on many different economically important performance characters of thousands, sometimes millions of animals. This information is processed using complex statistical procedures.

Reproductive technologies are used to spread genes from the best animals more widely and to turn over generations more rapidly. Dairy cattle improvement programs, for example, are entirely dependant on the technology of artificial insemination (AI) and the freezing of semen. This allows a bull to be evaluated accurately based on the performance of a large family of daughters. If he is selected on the basis of this information he can then produce many thousands of high merit daughters. Similarly, multiple ovulation and embryo transfer (ET) allow elite cows to produce many more progeny than would be possible through natural breeding (see articles on pages 30 & 32).

First Glimpses of the Genome

The success of modern genetic improvement to this point has been achieved without being able to look at the more than 70,000 genes that determine the unique genetic makeup of each animal. The explosive development of molecular genetic technologies are now beginning to give us our first glimpse into the genome. With rare exceptions, we are still a long way from being able to identify the functions of the 70,000 genes, and even farther from being able to define the optimum combination of variants at each of those genes.

The rare exceptions involve mutations of genes that cause major deleterious effects. An example is the discovery of the gene that causes porcine stress syndrome (PSS), where afflicted pigs are extremely sensitive to stress and have carcasses with poor meat quality (See article on pg. 43). The genetic test developed for this gene was the result of a collaborative effort between David MacLennan at the University of Toronto and Peter O'Brien at the University of Guelph, and is being used world-wide to improve pig welfare and meat quality by eliminating the mutant form from the population.

Marker Assisted Selection

Since defining the roles of the 70,000 plus genes is a long way off, animal scientists are currently hanging their hopes on genetic markers. These markers have no function of their own - they simply identify a particular region of genetic instructions. This region will contain hundreds of genes that do have a



DAIRY CATTLE SELECTION WITH MARKER ASSISTED AND IN VITRO FERTILZATION





Selected bull

3 month old heifer from an elite cow

TIME

• IVF and
• ET

many progeny born

9 months

• marker assisted selection

Selected bull

3 month old selected heifers go for IVF

• IVF and
• ET

12 months

male progeny born

21 months

• marker assisted selection

Young bulls selected for progeny testing at 1 yr old

33 months

• artificial insemination
• data collection and collation
• genetic evaluation technologies

Final selection based on progeny test results

72 months

function, but we do not generally know which genes they are or what is their function. But, we can follow the inheritance of many different markers in families of animals and see whether inheritance of any of these markers is associated with improved performance. If they are, we then know that one or more genes in the region of the marker are having a beneficial effect. We do not need to discover which genes are involved, but can use the information on the genetic markers to make future selection decisions, since animals that inherit the marker will also inherit the useful effects associated with it. This is known as 'marker assisted selection'.

The genetic marker tests can be done at any age, even on early embryos, so it is possible to select animals or embryos on the basis of this information prior to entry to conventional performance testing programs. The first Canadian project in this area, a collaborative venture between University of Guelph and the Saskatchewan Research Council, is just starting.

Existing reproductive technologies, such as artificial insemination and embryo transfer will help by producing larger families than currently required, which can then be selected for the best marker combinations. Emerging reproductive technologies such as IVF should allow large families to be produced from very young females, which opens the door to having one or two generations of marker selection prior to conventional selection, with little increase in time required (see sidebar).

Footnote to sidebar:

The final selection of bulls after progeny test takes place about one year longer than with conventional selection schemes. There have been two additional rounds of selection based on genetic markers, which means that considerably more progress is made per year with the new scheme.

* IVF = oocyte recovery and in vitro fertilization technologies

* ET = embryo transfer technologies

Selection of breeding animals with the most desirable traits and the dissemination of their genes through the population are key to increasing the productivity and profitability of dairy cattle. Genetic gain, the rate at which this occurs, depends on the intensity and accuracy of selection, and the generation interval.

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EMBRYO BIOTECHNOLOGY for the Canadian Dairy Industry



PHOTO: D.L.SHEPARD

Researchers Naida Loskutoff and Walter Johnson are shown with identical quadruplet bulls produced from a single four-cell embryo by *in vitro* fertilization.

The intensity of genetic selection for dairy breeding has vastly increased since the 1950s by the collection of semen from a limited number of elite bulls for artificial insemination (AI). In the 1970s, the development of multiple ovulation and embryo transfer (MOET) made it possible to significantly increase the intensity of selection of desirable genetic traits of elite cows, as well. The application of both AI and MOET in sophisticated programs of progeny testing has improved the accuracy of selection. More recent advances in the production, genetic evaluation, and manipulation of embryos have the potential to further improve selection intensity and accuracy, and, most notably, to reduce the generation interval.

The *in vitro* production of embryos (IVP) is an alternative to multiple ovulation. Oocytes (eggs) are collected from the ovary and matured and fertilized in the laboratory. The resulting embryos are cultured to the morula or blastocyst stage when they can be transfered to recipients. This approach is being applied commercially for the production of embryos from otherwise infertile valuable cows, and for the large scale production of embryos of desirable type, but not necessarily specific pedigree. Experience in the Netherlands and the USA suggests that IVP may be as, or more, efficient



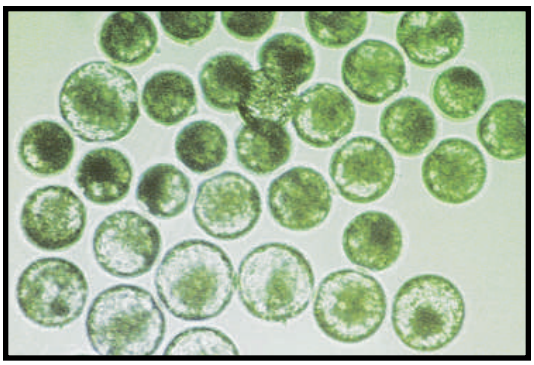
than multiple ovulation, even in normal cows. Furthermore, IVP offers a way to significantly reduce the generation interval, and provides embryos at very early developmental stages when they can be evaluated or manipulated to meet specific genetic goals.

The generation interval can be shortened by at least a year by using IVP to produce embryos from oocytes collected from juvenile or prepubertal heifers. The success rates are still relatively low, but oocytes can be collected every two weeks from heifers as young three to five months and yield an average of one pregnancy per collection. The use of IVP might also reduce the generation interval for bulls; the number of sperm that can be collected from bulls early in puberty may be inadequate for use in AI, but would be more than sufficient to produce hundreds of embryos by IVP.

"We have in Canada the expertise, the infrastructure, and a history of willingness to implement new techniques for genetic improvement. Consequently, the Canadian dairy industry is well situated to respond to changes in genetic requirements through embryo biotechnology."

A group of *in vitro* produced cattle embryos at seven days after fertilization.

PHOTO: KEITH BETTERIDGE



The accuracy of genetic selection is increased by both MOET and IVP because genetic variability is reduced by the production of groups of full sisters for progeny testing. The optimum would be to reduce or eliminate genetic variability altogether by producing clones of identical calves. Monozygotic twins and quadruplets have been produced by splitting early embryos. Larger clones have been produced by fusing single blastomeres taken from 8-16 cell embryos to enucleated oocytes, but this is technically more difficult.

Simple Non-Invasive Embryo Sexing

The ability to pre-select the sex of the offspring would ensure that either bulls or heifers could be produced, depending on the particular requirement of a given stage of the genetic selection program. The sex of early embryos can be determined by DNA analysis of a biopsy of one or a few cells. The recent development of antibodies against sex-specific antigens by researcher S. R. Blecher here at Guelph promises to allow the determination of the sex of an embryo without the time-consuming and invasive biopsy required for DNA sexing (see article on page 35). It is now also possible to separate X- and Y-chromosome bearing sperm in sufficient quantities for *in vitro* fertilization and thus produce embryos of only the desired sex.

Geneticists and reproductive physiologists have long recognized the potential importance of IVP, cloning and sexing to genetic improvement. These techniques have been relatively slow to develop but are now at, or very near, the point of practical application, and the increased rate of genetic gain that they offer is of immediate importance. For example, the increasing emphasis on the protein content of milk necessitates a significant change in dairy genetics if Canadian milk producers and dairy breeders are to compete in the world market. Fortunately, we have in Canada the expertise, the infrastructure, and a history of willingness to implement new techniques for genetic improvement. Consequently, the Canadian dairy industry is well situated to respond to changes in genetic requirements through embryo biotechnology.

"It is impossible to exaggerate the influence of cryobiology on the cattle industry over the past 25 to 30 years."

CRYOBIOLOGY

Consider just two examples. Frozen bull sperm used for artificial insemination (AI) has permitted intense selection to identify those few bulls in a dairy herd whose daughters produce the largest quantities of the highest quality milk. Once such a prize bull is identified, thousands of 'doses' of his semen are collected, frozen in liquid nitrogen at -196°C and stored in the frozen state for months, years or decades. The frozen sperm can then be distributed all over the world to be used for AI.

In Canada, selection of the best bulls has meant that 800,000 dairy cows required to meet domestic market demand for milk in 1951 could be decreased to 443,000 by 1991 (Vol. 17 No. 2). This 45% reduction in the number of dairy cows represents an enormous increase in the efficiency of milk production. As a result, millions of dollars are generated by the collection and sale of frozen bull sperm.

A second example is found in the field of embryo transfer (ET). For the last 20 years, cattle embryos have been cryopreserved for ET. This has significantly increased the efficiency of ET, because it reduces logistical problems of matching available embryos with suitable recipient foster cows. In Canada, many thousands of dairy cattle embryos are now cryopreserved for transfer either in Canada or in many other countries. At least 75% of the dairy bulls in the selection process described above were themselves ET calves. Despite this success, there remain opportunities for improvement of these two applications of cryobiology to the dairy industry. Research at the University of Guelph is furthering the techniques of cryobiology for agricultural and biomedical research purposes.

Improvement of Bull Sperm Cryopreservation

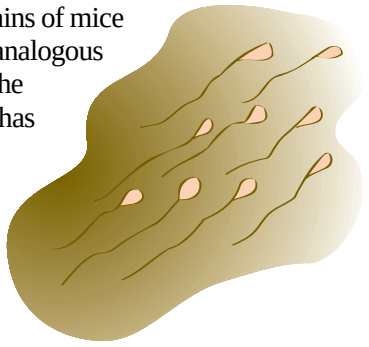
About 50% of the sperm of most bulls are damaged by current methods of freezing; 25% of the ejaculates of some bulls are unusable after freezing because too few sperm survive. Our research is identifying cryoprotectants to yield higher sperm survival after freezing. We are searching for the optimum freezing and thawing rates that will yield the highest possible survival of sperm, and methods to reduce or eliminate differences in survival of sperm from different bulls.

A Rapid Freezing Method For Bull Sperm

Sperm, like many types of mammalian cells, experience chilling injury when cooled to temperatures close to 0°C. The longer they are exposed, the more sperm are damaged. Standard methods of freezing are rather slow, thus exposing the sperm to damaging temperatures for several minutes. An alternative would be to cool the sperm rapidly, thereby passing them through the zone of damaging temperatures rapidly enough to avoid injury. Experiments at Guelph indicate that this approach may be faster, more efficient, and more reproducible than current slow-freezing methods.

Cryopreservation of Mouse Sperm

Transgenic mice have become enormously important in the study of both human and animal diseases, and in the development of new therapeutics. However, the number of new strains of mice required for this purpose are exhausting mouse breeding facilities. An alternative would be to 'bank' new strains of mice by freezing their sperm, in a way analogous to that described for bull sperm. The U.S. National Institutes of Health has recently awarded research grants totalling more than $1 million (U.S.) to develop a method of freezing mouse sperm.



A new method devised in the Cryobiology program at

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FOR ONTARIO AGRICULTURE



PHOTO: CARROLL AND CARROLL

Guelph has already produced more than 15 litters of mice derived from cryopreserved sperm. This should find wide applicability in the husbandry of laboratory animals. It will also substantially reduce the number of live animals required for certain types of investigation.

Cryopreservation of Bovine Oocytes by a Novel, Ultra-Rapid Cooling Procedure

Until now, methods available to cryopreserve bovine eggs yielded only a few percent survival, too low for practical application. By an innovative method recently developed at Guelph, 15% of cryopreserved oocytes developed into blastocysts after in vitro fertilization (IVF), a rate close to half that of unfrozen control oocytes. When this method of oocyte cryopreservation is fully developed, it will permit much more flexible pairing of the most valuable bulls and cows by IVF of cryopreserved eggs with frozen sperm. It will also allow 'retrospective mating' of dams and sires, enabling cattle breeders to capitalize on the best features of animals, even long after their deaths.

Improvement of Cryopreservation of *In Vitro*-Derived Bovine Embryos

At Guelph, as at many institutions around the world, cattle embryos are now routinely produced by IVF of eggs collected from ovaries of cows being slaughtered for beef. These in vitro-derived embryos are beginning to be used commercially as a cheaper and more reliable source of bovine genetics for embryo transfer. Companies in Canada, the United States, The Netherlands, France and Japan already sell millions of dollars worth of such IVF embryos. Unfortunately, these in vitro-derived embryos have generally been more difficult to cryopreserve than their in vivo-derived counterparts.

Research at Guelph has recently produced two findings to significantly improve the survival of in vitro-derived embryos. First, altering the culture conditions used to grow IVF embryos has not only yielded higher survival of day seven embryos, the most common stage for freezing embryos, it has also permitted day six embryos to be successfully cryopreserved. This has not been possible before.

The procedures used to cryopreserve IVF embryos, for direct transfer under field conditions, have also been improved. Close to 100% of embryos frozen in this way survive, and 16 pregnancies have now been produced.



Cryopreservation of Chicken Blastoderm Cells

Blastodermal cells are the vehicle through which specific genetic alterations can be made to the genome of chickens, using the tools of molecular and cellular biology (see Fig. 1. on page 37). As lines of genetically altered blastodermal cells are developed and maintained in vitro, insertion of these cells into chimeras (see Fig. 2. on page 38) would be made easier by the ability to freeze and thaw the cells. Freezing and thawing cells has also created a new opportunity for gene banking that is currently being discussed by an international task force. Collaboration between Robert Etches' team in the Department of Animal & Poultry Science and those in the Department of Biomedical Sciences at Guelph has produced the first chicken that has been 'reconstituted' from cells stored at -196°C. Further development of this technology would facilitate the production of transgenic chickens in the long term. In the short term, the ability to hold breeds and strains as frozen blastodermal cells can be applied to conserve the enormous genetic diversity that is threatened by the expense of maintaining live populations of chickens.

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Reproductive BIOTECHNOLOGIES for SUPERIOR ONTARIO SWINE genetics

Acknowledgments: This work has been supported by Struthers Research Inc., NRC and OMAFRA

New reproductive biotechnologies are currently under development in the Department of Population Medicine for improved swine-breeding strategies. This work, part of the Struthers Research project, is centered on production of offspring from gilts, following slaughter and carcass evaluation. Specific technologies include oocyte transfer, *in vitro* embryo production procedures, short and long term embryo storage systems and non-surgical embryo transfer. Commercially, these new methods could lead to direct selection for improved meat quality in the pork industry, and significant reductions in the interval between generations under selection.

Embryo, Oocyte Transfer, and *In Vitro* Embryo Production

Reproduction from superior carcasses after slaughter must be technologically reliable for success. Because only one opportunity exists for the production of viable embryos using slaughtered animals, we have developed procedures for embryo transfer, oocyte transfer and *in vitro* embryo production from ova recovered from slaughtered gilts (Fig. 1).

Oocyte recovery and transfer procedures have lead to the birth at Guelph, of North America's first piglets produced through oocyte transfer. Techniques have been developed for both transvaginal collection of oocytes from living sows and for laboratory recovery of oocytes from slaughtered gilts. These studies have demonstrated that large numbers of oocytes may be recovered from superovulated gilts after slaughter. Following development of oocyte recovery techniques, the efficacy of oocyte transfer methods was tested. Taking our lead from procedures for treatment of human infertility, protocols have been elaborated for the transfer of oocytes to the oviducts of synchronized sows. Our work has shown that both an acceptable pregnancy rate and live offspring can be obtained by these methods.

New reproductive biotechnologies would benefit both commercial pork and seed stock producers in Ontario. Development of atraumatic embryo recovery and transfer techniques, methods for short and long term embryo culture, and procedures which allow for the creation of viable embryos from gametes harvested from slaughtered animals will increase the use and sales of superior Ontario swine genetics in Canada and abroad.

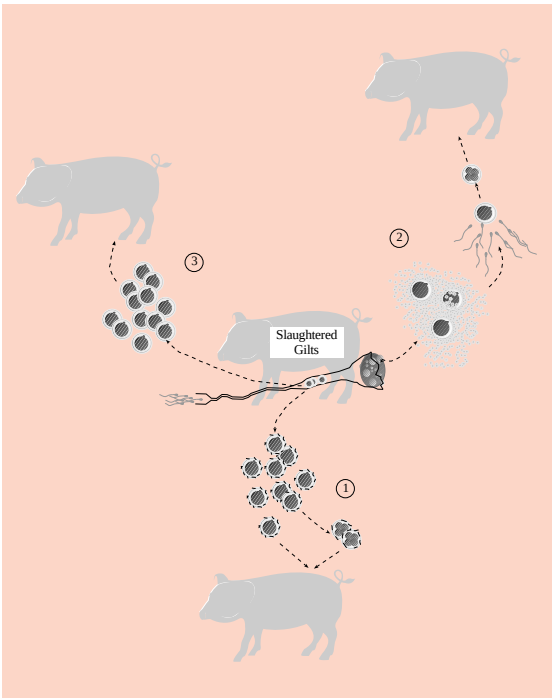


Fig. 1. Application of reproductive biotechnologies developed at the University of Guelph, to swine breeding. (1) embryo transfer, (2) oocyte transfer, and (3) *in vitro* embryo production.

Antibodies

Put Embryo Sexing on Target



PHOTO: DAVID BARR

Beef producers prefer male calves for their industry. Recent results from our laboratory at the University of Guelph suggest it will be possible to develop a commercially viable, non-invasive embryo sexing method to produce embryos of the preferred sex, by using antibodies to target male or female embryos. It is a different approach than has been tried in the past to sex embryos.

Sex-specific Proteins are Key

Embryos, as well as fetuses and adult animals of each sex, contain sex-specific proteins (SSPs). They're present on the outer surface of many cells, including embryo cells. It is possible to prepare antibodies that zero-in on SSPs, selectively eliminating the female embryos and leaving the male embryos untouched. In laboratory tests, it has been possible to obtain pure cultures of male embryos with 98 per cent accuracy. Embryos selected in this way can be recovered and the antibodies washed off; then the embryos can continue growing in a laboratory until they're large enough to be transferred to a cow.

A Non-Invasive Technique

A very positive aspect of this method is that it requires minimal human intervention, unlike other techniques such as DNA analysis. Using the DNA of embryos to determine their sex has been successfully accomplished, but it is an invasive procedure that requires that the protective covering of the embryo be opened and a few embryonic cells removed to obtain the DNA. The embryo may not grow well after some of its cells have been removed and breakage in the covering may lead to infection. DNA removal is also complex and requires specialized equipment, so it is not widely available for field use.

Another method of sexing embryos – chromosome removal and analysis – is likewise complex, time-consuming and possible only by highly-skilled technicians.

Efforts to use antibodies to SSPs to determine sex have been attempted elsewhere in the world, with limited success. However, none used purified SSPs as a starting point, which is unique to our work.

Our method involves extracting SSPs from bovine organ tissue obtained from the slaughterhouse. The tissues are ground very finely and the cell membranes that form the outer covering of the cells are recovered. Male and female SSPs are then obtained from the cell membrane samples using biochemical procedures such as chromatography and gel filtration. The result is purified SSPs.

Potential to Sex Sperm Cells

Antibodies are prepared by injecting SSPs into laboratory animals of different species, such as rabbits. The animals produce antibodies to the SSPs; blood samples then provide the antiserum which includes the antibodies. The antisera can be tested to determine if the antibodies successfully target male or female embryos, and whether they could similarly target sperm cells containing the Y or X chromosome, another potential application for this research. Preliminary results show it is possible that our method could be modified to sex sperm cells.

The nearly total accuracy achieved in the laboratory in targeting male embryos demonstrates the feasibility of the procedure. The methodology for mass producing SSPs has been worked out. Now, with further work, the procedure can be transferable to the agricultural community and developed for females, as well, for the benefit of the dairy industry. This would greatly reduce the loss that occurs through culling the unwanted sex in beef and dairy farming. As well, it could enhance the effect of livestock genetic research in general, by increasing the rate at which animals of the required sex would be available for breeding.

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TRANSGENIC Chickens:

Promising Applications for Ontario's Poultry Industry



Chickens have been domesticated for about 4000 years, and bred for a diverse array of genetic differences. Today, thousands of breeds and varieties are recognized at exhibitions, maintained by academic institutions, and disseminated by poultry breeding companies. The techniques available to assist the poultry breeder have changed radically during the history of domestication. For the first 3,500 years, selection was practised on morphological traits that could be easily recognized, such as size, body conformation, fatness, feather color, comb type, fighting ability, feather structure, etc.. As biology became a more quantitative science, breeders classified stocks on their ability to grow at precise rates, to lay a precise number of eggs, to begin laying at a precise age, and to convert feed to poultry products at precise values.

Now, as the tools of biotechnology are applied to poultry breeding, we are learning how to selectively alter genes that are believed to affect performance and to assess the accuracy of the predictions.



Chimeric Chickens: A First Step

The key to making specific genetic alterations is to gain access to cells that will subsequently form the ova and sperm. In birds, these cells are present in an early stage of embryonic development when the embryo is a blastoderm. Studies conducted during the past five years by members of my laboratory and that of Ann Gibbins' have shown that cells contained within the blastoderm can give rise to the germline of chimeras. Chimeras are made by injecting approximately 500 blastodermal cells from a donor embryo into a recipient embryo at the same stage of development.

In our experimental work, we have color-coded the donors and recipients by using Barred Plymouth Rocks (a black breed) as donors and White Leghorns (a white breed) as recipients. Chicks that hatch with splashes of black and white down, therefore, contain cells derived from the Barred Rock donor and from the White Leghorn recipient, respectively. Approximately 50% of the chickens that are feather-color chimeras also contain donor-derived cells in their testes or ovaries and produce offspring that are derived from the donor cells. This transmission of the donor-derived genome to the next generation is the fundamental attribute of chimeras. It's also why they can be used to produce transgenic chickens.

Chimeras can carry genetic alterations to the next generation by inserting foreign DNA into donor cells that are destined to become sperm and eggs, and then injecting the genetically modified cells into recipient embryos (Fig. 1). If genetically modified cells colonize the germline of the chimera and produce genetically modified sperm, the genetic modification will be transmitted to the offspring. We are currently evaluating the inheritance of several genes that have been introduced into blastodermal cells, and subsequently been used to make chimeras.

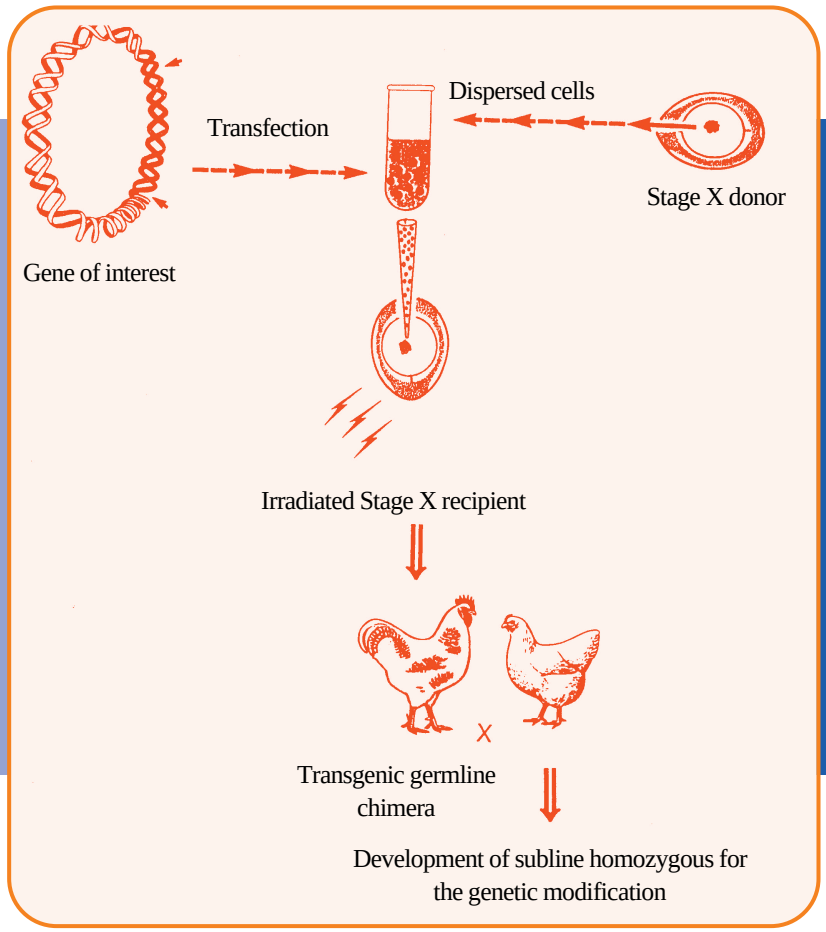


Fig. 1. Transgenic germline chimeras that can transmit a genetic modification to the next generation are made by (1) collecting blastodermal cells from a stage X Barred Plymouth Rock donor embryo. Embryos at this stage of development are found in newly laid, fertile, unincubated eggs. (2) Introducing the gene of interest into these cells by a process called transfection. (3) Injecting transfected cells into irradiated stage X recipient embryos. Irradiating the recipient provides a host embryo that augments the contribution of donor cells to the chimera. (4) Hatching chimeras that can be recognized at hatch by their black and white down. (5) Breeding chimeras to determine if they have transmitted the genetic modification that was introduced in step 2 to their offspring. Sublines that express the genetic modification can then be developed from this foundation stock.

Transgenic Chickens: Benefits for Human Biology

Candidate genes for the production of transgenic chickens must be reasonably well characterized at the molecular level. For example, a family of genes regulating early stages of development have been recognized in organisms as diverse as fruit flies and man. One of the remarkable aspects of these genes is the similarity in their nucleotide sequences, regardless of the animal in which they reside.

The actions of this family of genes is not well understood, however, because there are few good vertebrate models in which they can be studied. Transgenic chickens offer an attractive opportunity to study the effects of altering expression of these genes and to deduce their function from the consequences.

In the poultry breeding industry, there are promising applications of transgenic technology. Several genes are known to alter resistance to disease and strategies have been suggested for the production of disease resistant chickens. In mice, the array of digestive enzymes that are produced within the gut has been enhanced using transgenic technology and similar applications can be imagined in poultry. Chickens resulting from these breeding programs might be more able to digest feedstuffs that are not otherwise useful to the humans. Transgenic technology might also produce chickens that deposit novel proteins in their eggs, which could then be harvested from eggs and used in medicine or manufacturing processes. The use of chickens to produce novel therapeutic proteins is discussed more extensively in the accompanying article by Ann Gibbins (see page 39).

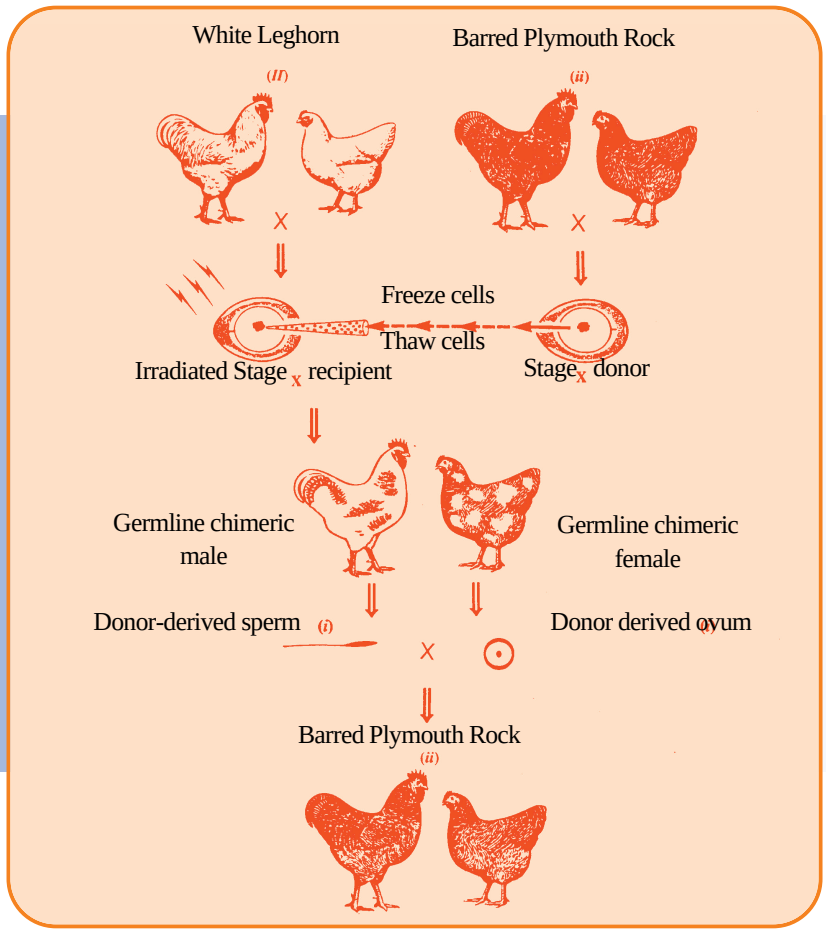


Fig. 2. The genome of a breed, strain or genetically defined group of chickens can be stored as frozen blastodermal cells. Blastodermal cells are harvested from stage X embryos, frozen and stored for an indefinite period. The cells are then thawed and injected into recipient embryos to form chimeras. The chimeras are mated together and reproduce the original breed when a donor-derived egg is fertilized by a donor-derived spermatozoa.

The Importance of a Gene Bank

The poultry breeding community currently faces a dilemma created by their success in having bred such a vast array of genotypes from their wild ancestors, because of the costs of maintaining these diverse stocks. Until the advent of germline chimeras, the only way to maintain a purebred stock was to keep males and females of the line as live birds. During the past year, however, a visiting scientist from Japan, Katsutoshi Kino, and Stan Leibo from the Department of Biomedical Sciences and members of my laboratory have demonstrated that blastodermal cells retain their ability to contribute to the sperm and eggs of chimeras, even after freezing and thawing (Fig. 2). This discovery has created an opportunity to hold lines of chickens as cryopreserved blastodermal cells. When the line is required in the future, the frozen blastodermal cells would be thawed and injected into recipient embryos to make germline chimeras. Matings between germline chimeras would produce chicks of the original line. Adoption of this technology could prevent the disappearance of unique lines of chickens from cost cutting public institutions that have been the repositories of this genetic heritage.

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Chickens as Bioreactors

Because of remarkable developments in genetic engineering during the past 15 years, foreign proteins are now being produced in 'bioreactors' following introduction of their modified genes into animals, plants, microorganisms and cultured cells. For example, most commercially available human insulin is now synthesized artificially in microorganisms, and several human proteins are being manufactured by transgenic plants and purified from harvested seeds or leaves.

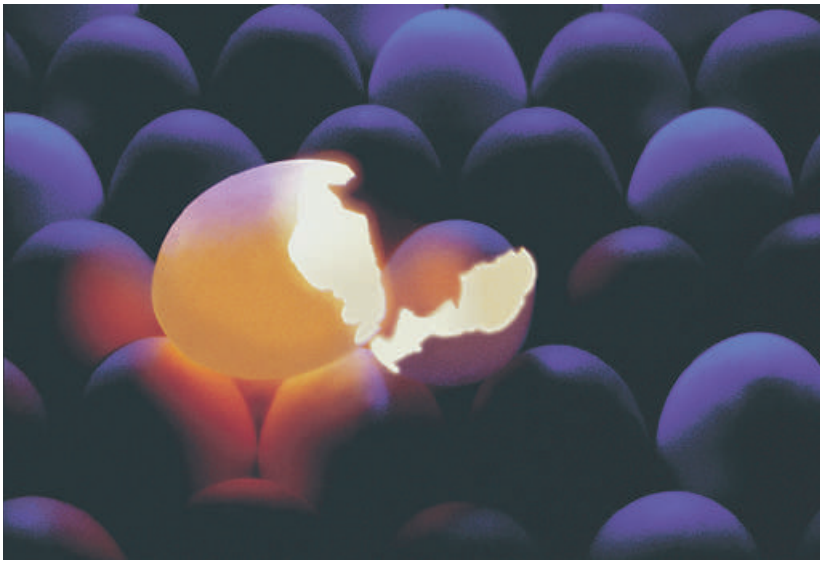


PHOTO: MASTERFILE/ PAUL TERPANJIAN

HARVESTING COMMERCIALLY-VALUABLE PROTEINS FROM THE EGG

The capability of domesticated mammals such as sheep, goats and cows to produce substantial quantities of proteins in milk has been put to good use – some transgenic livestock now contain genes encoding therapeutic human proteins. These genes have been altered to ensure the deposition of the corresponding proteins in milk, from which they can be isolated easily. Examples of proteins being produced commercially in this way by companies such as Genzyme Transgenics, PPL Therapeutics and GenPharm International include lactoferrin (for prevention of gastrointestinal infection), collagen (for tissue repair), alpha-1-antitrypsin (to treat emphysema), and fibrinogen (as a tissue adhesive).

Some of the foreign proteins produced by bioreactors, such as human insulin, may be equivalent to those found naturally, but may normally be produced in minute amounts or may be difficult to isolate from their usual source, especially in a pathogen-free state.



PHOTO: L.NORMAN GIBBINS

Photo: With the production of transgenic chickens now possible, through a chimeric intermediate (here constructed by injecting genetically manipulated and sorted Barred Plymouth Rock donor embryo cells into a White Leghorn recipient embryo), work can now focus on producing transgenic birds that deposit novel products in the egg.

Alternatively, the foreign proteins may be completely novel, with no natural counterpart. Our increased understanding of gene and protein structure is allowing the molecular geneticist to custom-design genes and proteins and to predetermine not only where and when a foreign gene will be expressed in a bioreactor organism, but also how the resulting protein will function. Surprisingly, we can indeed improve on Nature in our quest to produce proteins that have therapeutic or commercial value.

An Ideal Container for Deposition of Novel Proteins

One highly productive agricultural species that has been bypassed in designing novel approaches to the synthesis of bioreactive proteins is the chicken. The egg could provide an ideal container for the deposition and storage of foreign proteins, with no detrimental effect on the hen. Because of the relatively short generation time of the chicken and the large number of progeny, genetically-manipulated birds could be introduced into commercial breeding programs far more rapidly than could most transgenic mammalian livestock animals. What has been lacking until now has been an effective method for producing transgenic birds, but, as described by my colleague, Rob Etches, in the accompanying article (see page 36), we and our team of researchers have been successful in producing transgenic birds through a chimeric intermediate, following the transfer of foreign genes to chicken embryo cells. For example, the photograph shows a chimeric chicken held by Gord Speksnijder, one of the graduate students working with me, who constructed this bird by injecting genetically-manipulated and sorted Barred Plymouth Rock donor embryo cells into a White Leghorn recipient embryo. We are now ready to focus our activity on producing transgenic birds that deposit novel products in the egg.

What types of chicken bioreactor might be produced? Normal components of the egg could be modified to increase their quantity or to improve their functional properties. More dramatically, totally novel proteins such as human therapeutic proteins could be deposited in the egg.

Genes consist of two essential components – the so-called *structural* sequences, which contain the genetic code for the protein that is to be produced, and the *regulatory* sequences, which dictate where and when the gene will become active and manufacture a product. Egg proteins are found in two main compartments, the yolk or the white – most egg yolk proteins are synthesized in the liver and make their way to the ovary through the blood stream. In the ovary, they are transferred specifically to oocytes, which will form the future yolks. Egg white proteins are synthesized in the tubular gland cells of the oviduct, and are deposited as components of egg white while the egg moves through the magnum prior to being laid. Knowledge of genes encoding egg yolk and white proteins could be tapped to ensure that novel proteins were deposited in the egg.

For several years, graduate students in my laboratory – most recently Cynthia Millan and Guodong Liu – have studied the regulatory regions and specific modifications of the vitellogenin gene, vitellogenin being a major component of egg yolk. More recently, another student, Jan Losos, has begun to transfer modified versions of the lysozyme gene to chicken embryos, lysozyme being an important component of egg white. Thus, we are preparing to modify the contents of both egg yolk and white.

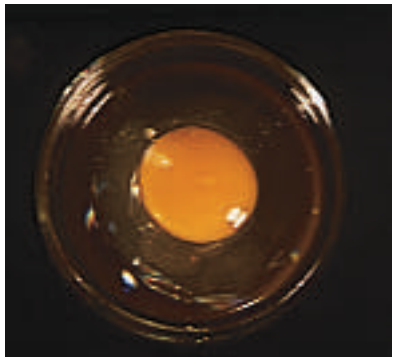


PHOTO: L. NORMAN GIBBINS

Using the chicken as a bioreactor, normal components of the egg could be modified to increase their quantity or improve functional properties. More dramatically, totally novel products such as human therapeutic proteins could be deposited in the egg.

Production of Enhanced Bactericidal Lysozyme

Apart from the value of egg preparations and whole eggs as basic foodstuffs, several normal components of chicken eggs are commercially valuable and could be improved by genetic manipulation. An example is lysozyme, which constitutes only 3.5% of egg white proteins and yet is used widely in the Pacific Rim countries as a bactericidal agent. Canada is the major manufacturer of lysozyme and the market is by no means saturated – an increase in the amount of lysozyme deposited in the hen's egg would be welcomed by industry, and should have no detrimental effects on the bird. An exciting possibility is the genetic manipulation of the lysozyme gene to give an enzyme that is effective against more bacterial species than are attacked by natural lysozyme. A growing concern in the medical community is the increased resistance of pathogenic bacteria to the current range of antibiotics, and there is heightened interest in developing other antibacterial agents such as modified forms of lysozyme.

In my laboratory, we have begun a series of experiments in collaboration with Albrecht Sippel of the University of Freiburg, Germany, in which we are inserting extra copies of the lysozyme gene into chickens to improve lysozyme production. Next, we could insert altered copies of the gene to produce lysozyme with different antibacterial capabilities. The lysozyme gene has several unique capabilities – firstly, it has powerful regulatory regions that ensure the synthesis of plentiful amounts of product. In addition, the natural gene is bordered by special DNA sequences named MAR elements (matrix attachment regions), which insulate the gene from potentially repressive influences of neighbouring regions of DNA in the chromosome. In other words, the regulatory regions of the lysozyme gene could be used as an ideal framework to carry structural regions of genes encoding other bioactive proteins produced in quantity and isolated from egg white. We plan to pursue this opportunity.

Acknowledgement: We are grateful for financial assistance from the Ontario Egg Producers' Marketing Board, OMAFRA, NSERC, URIF, NATO, Agriculture Canada, and the University of Guelph Research Enhancement Fund.

Custom-Designed Antibodies to Prevent Gastroenteritis

As far as novel egg yolk proteins are concerned, Cynthia Millan and I have been interested in the intriguing possibility that transgenic hens could be constructed that would synthesize custom-designed antibodies. In common with mammals, birds provide their young with maternal antibodies to combat infection before the immune system of the young animal is functional. Most avian maternal antibodies are sequestered by oocytes in the ovary, and eventually are deposited in the yolk compartment of laid eggs. This rich source of specific antibodies has been exploited by the deliberate immunization of hens with a wide variety of antigens; the resulting antibodies can be isolated from the yolk in highly purified form and at high titre.

The advantages of raising antibodies in birds as opposed to mammals are numerous, but one attractive possibility is that chicken antibodies could be valuable prophylactic agents in the prevention of gastroenteritis in humans, livestock animals and pets. They could be consumed in the form of the whole egg or yolk as part of a normal diet, especially since antibodies in eggs appear to be relatively resistant to the heat employed in cooking and to acidic conditions similar to those in the intestinal tract.

The production of specific chicken antibodies in immunized hens is not novel. But the concept that Cynthia Millan and I believe should be implemented is that of custom-designing hybrid genes encoding antibody-like peptides, reactive against specific pathogens in specific hosts (for example, humans, pigs or cattle), yet retaining some of the superior characterisitic of chicken antibodies – and still deposited in eggs. Each laboratory-designed antibody gene would be used to construct a line of transgenic chickens. In this way, antibodies would be produced that could never be synthesized by normal birds, not even if these were specifically immunized.

Many aspects of this concept are coming to fruition – highly engineered mammalian antibody genes bearing little resemblance to their natural counterparts are now being expressed in bioreactor systems as diverse as transgenic plants and microorganisms – why not expect chickens to serve as an alternative host in the future, with all of the unique biological and commercial advantages that this system could afford?

Identifying Boar Taint Genes

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In North America, all male pigs raised for meat are castrated early in life to prevent taint in the carcass when they reach market weight and near sexual maturity. But castration also removes the source of the natural growth promoting androgens that allow males to utilize feed efficiently and produce lean carcasses. In some countries, such as Great Britain and Australia, pigs are not castrated since they are traditionally marketed at light weights when boar taint is low. However, at the heavy market weights used in Canada and the U.S., boar taint would be present in 5-20% of uncastrated male pigs.

Boar taint can be caused by high levels of either skatole or androstenone in the fat. Skatole is produced in the pig's digestive tract by bacteria which degrade the amino acid tryptophan. Androstenone is a steroid produced in the testis as the animal nears sexual maturity.

It might be possible to sort out the tainted carcasses, but rapid methods for measuring taint which could be adapted to the line speeds of modern packing plants are not yet available. It would thus be more desirable to raise uncastrated male pigs that were free of boar taint. This could be possible if genetic markers for low boar taint were developed for use in breeding programs.

Genes for Skatole

Skatole absorbed from the gut is metabolized in the liver and excreted. Skatole levels are higher in males and this might be due to increased absorption from the gut or decreased metabolism in the liver. During a recent sabbatical with Kerstin Lundström at the Swedish University of Agricultural Sciences, we studied the metabolism of skatole in the liver by the cytochrome P450 enzyme system.

Males which have high levels of skatole in fat had lower levels of a particular isoform of P450 in the liver known as cytochrome P4502E. This is shown in Fig. 1, where cytochrome P4502E is identified by reaction with specific antibodies using the technique of Western blotting. Our task now is to develop genetic markers to identify pigs with high levels of P4502E in liver which will not be tainted from skatole.

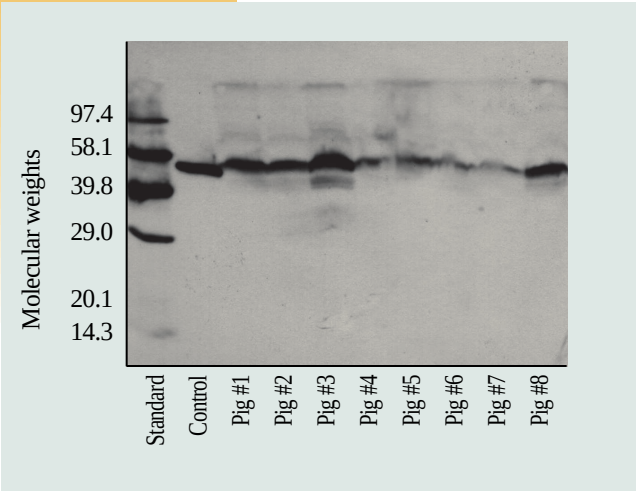


Fig. 1. Cytochrome P4502E, the protein responsible for metabolism of skatole in the liver, is indicated by band intensity, in samples of eight pig livers. Pigs with high levels of P4502E in the liver will not be tainted from skatole.

Genes for Androstenone

Over the past few years, we have studied the synthesis of androstenone in pig testis. We have shown that this also involves the cytochrome P450 enzyme system, specifically cytochrome P450c17, which is also responsible for the synthesis of the androgens. However, the synthesis of androstenone also requires the presence of another protein known as cytochrome b5. Again, using the technique of Western blotting, we have discovered that levels of cytochrome b5 in the testis are decreased in pigs with low levels of androstenone. We are now working to develop genetic probes to identify pigs with low levels of androstenone taint by identifying those animals that have low levels of cytochrome b5 in the testis.

Animal Welfare and Economic Benefits

If boar taint can be eliminated by selective breeding programs, it will no longer be necessary to castrate male pigs. Raising uncastrated male pigs could have substantial economic benefits to pork producers by decreasing feed costs and increasing lean meat yield. In addition, animal welfare concerns about castration of farm animals would be alleviated.

Acknowledgement: This work was supported by grants from OMAFRA, Ontario Pork and the Swedish Department of Agriculture and Forestry.

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Stressed Pigs

Get Better Fitting Genes

PHOTO: CARROLL & CARROLL



The modern pig has been bred to be faster growing, leaner, more feed efficient and to put pork on the dinner plate at a lower cost than pigs 30 years ago. This has lead to substantial benefits in terms of a healthier product with less waste and a lower use of natural resources (see Vol. 16 No.4). During the early 1970s, however, it became clear that these improvements were being made at the price of an increased frequency of pale, soft and watery meat, and an increased frequency of pigs with a peculiar sensitivity to stress.

Early family studies suggested that these effects might be caused by a single gene, but were not conclusive. Then, quite by chance, it was discovered that a few pigs anaesthetized with the gas halothane would rapidly become stiff and rigid while most pigs remained perfectly relaxed. Studies of this effect showed that it was controlled by a single gene, that it was pigs carrying two copies of a mutated form of this gene that turned rigid under halothane anaesthetization. The same pigs were stress susceptible and had pale, soft, watery meat. Later research showed that they were also faster growing and leaner. There was also a suggestion, but it could not be proved, that pigs which carried one copy of the mutant form of the gene were slightly faster growing and slightly leaner than normal pigs, even though

The Various Aliases of the Pig Stress Gene

This gene is known by many names reflecting the remarkable range of effects it causes, and the fact that for many years it was not known that all these effects were caused by a single gene.

The *PSS* gene: for Porcine Stress Syndrome. Pigs having two copies of this gene exhibit extreme, often fatal, susceptibility to stressful environments.

The *PSE* gene: for pale, soft exudative, referring to the pale, soft, watery meat that is produced by pigs that have two copies of this gene.

The *Halothane* gene: referring to the fact that the first test of this gene was that pigs carrying two copies of this gene go rigid when anaesthetized with halothane gas, while other pigs remain relaxed.

The *Ryanodine Receptor* gene: the mutation was eventually shown to be in this gene. The ryanodine receptor sits on the surface of muscle cells and is involved in maintaining one aspect of chemical balance inside the cells. It receives control instructions from a signalling molecule, known as ryanodine.

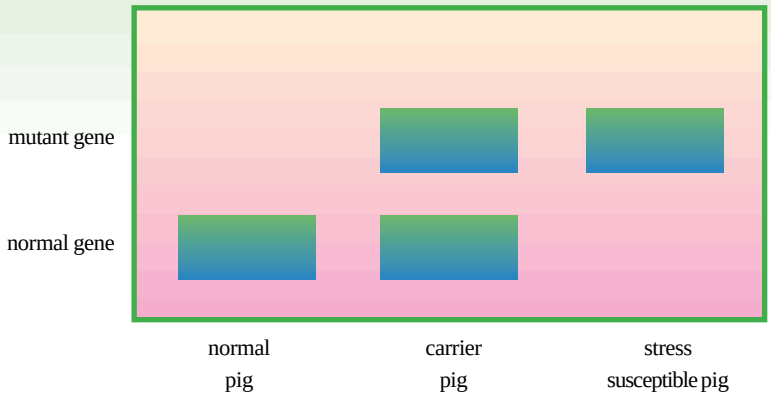


Fig.1. Molecular test for stress susceptability gene. Using the HAL-1483 test, the normal and mutant forms of the gene produce DNA fragments with slightly different lengths. With electrophoresis, the two fragments move to two different positions. With appropriate labelling, these can be seen as two clearly separated bands on the gel, as shown above.

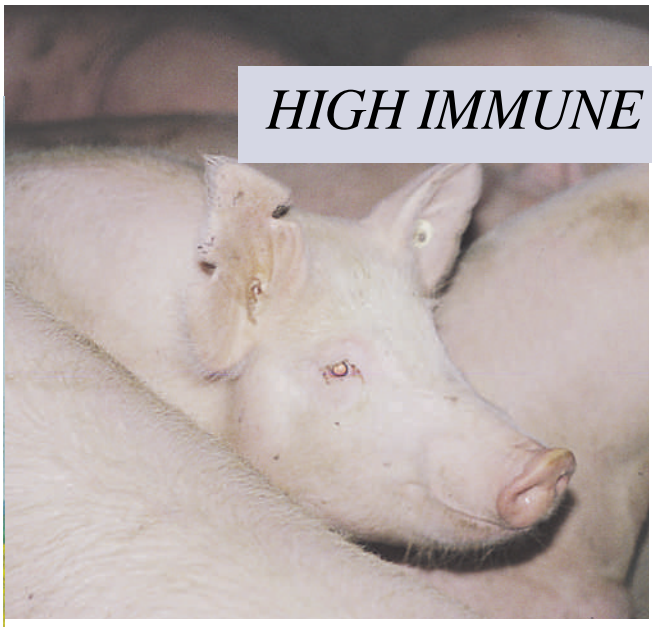
they did not show increased stress susceptibility and were not sensitive to the gas halothane. It was probably because of these positive effects that the mutant gene had increased in frequency in the populations so rapidly. Having discovered the gene, many pig breeders wanted to eliminate it or, at the very least, stop it from increasing further in their populations. The halothane test was, however, far from perfect since it only detected those pigs that had two copies of the mutant form, while the carriers with one copy (which were much more frequent in the population) remained undetected.

By the late 80s, several groups around the world suspected that the ryanodine receptor gene (see sidebar) might be the cause of a rare form of sensitivity to halothane in humans. David McLennan of the University of Toronto and Peter O'Brien of the University of Guelph collaborated to see if the same gene might be involved in halothane sensitivity in pigs. It was almost immediately shown that one particular mutation was the cause of halothane sensitivity in local pigs, and that this same mutation causes halothane sensitivity in all breeds of pig worldwide. This allowed the development and patenting of the HAL-1483® test for pigs which carry the stress susceptibility gene (Fig. 1). The availability of this test has made it possible to study the effects of this gene in much more detail. For example, as part of the Ontario Pork Carcass Appraisal Project, some 3,000 Ontario pigs with detailed information on growth, carcass composition and carcass quality are being tested for the gene. The results will provide accurate estimates of its effects on a wide range of commercially imported traits. On a direct practical level, the availability of the test has allowed pig breeders worldwide to detect and eliminate carriers of the gene in their population, thereby eliminating the stress susceptibility syndrome and improving the welfare of commercial pigs, as well as making substantial improvements in meat quality.

Acknowledgement: Research funded by Ontario Swine Improvement, Ontario Pork Producers Marketing Board and OMAFRA. Current testing for this gene in our experiments is in collaboration with VITA-TECH Canada Inc. HAL-1483® is a registered trademark of Innovations Foundation, Toronto.

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HIGH IMMUNE RESPONSE

PIGS

healthier, cheaper, faster

High immune response pigs at Arkell Research Station were developed based on a series of heritable (phenotypic) markers, indicating the animals' antibody response and cell mediated immunity. The markers were combined in a multi-trait selection index to produce a line of pigs with resistance to a wide range of pathogens.

The animal production industry has traditionally put great stock in breeding for production efficiencies and quality, using directly measurable traits such as weight gain, numbers of offspring, feed conversion efficiency and backfat.

Guelph researchers have now found a unique way to breed for both animal productivity and health, based on an animal's overall ability to resist disease. In purebred testing systems, the approach has resulted in major improvements in health and production performance.

The development is called high immune response (HIR) technology. Like traditional breeding strategies, it involves identifying animals bearing the required traits and crossing them to create superior offspring. But HIR animals also have a broad-based ability to resist disease.

HIR animals are selected based on efficiency of their immune systems. A series of heritable markers of the immune system have been developed and combined in a multi-trait selection index. The markers are phenotypic markers of the performance of antibody response and cell mediated immunity. Both functions are required for resistance to a wide range of disease-causing pathogens. Young pigs' response to immune-stimulating agents is measured by specific blood and skin tests.

With HIR animals, there's increased uniformity with respect to vaccination. Inherently healthier animals likely means fewer medications - good news from a production and environmental viewpoint.

An added bonus is that the animals' superior health also results in greater production. In test herds at the Arkell Research Station, HIR lines required 10 per cent fewer days to reach market weight. They also produced more piglets per litter. It's estimated that the lifetime incremental benefit per F_1 gilt in a production unit could be more than $400.

Through the efforts of GUARD (Guelph Alumni Research & Development), an Ontario consortium is taking the new technology further for commercialization. A project team including breeding strategists has been assembled to design rigorous evaluations in commercial herds.

Inherently Healthier HOLSTEINS

A selective breeding program at OVC is aimed at providing healthier livestock through selective breeding. Preventative methods of health management, such as selection for superior immune response and disease resistance, are known to be more economical than treatment of disease.

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To evaluate the potential of breeding disease resistant cattle, the research is examining genetic markers of disease resistance, enhanced immune function and innate resistance. A novel combination of traditional and marker assisted selection is being used. Major histocompatibility genes and other markers are being considered. Markers such as somatic cell counts alone may not be good selection criteria for reducing diseases such as mastitis, since they are indicators of infection, rather than resistance. Currently, over 1500 Holstein cows from 100 commercial or research herds in Ontario are being screened for useful genetic and immunological markers of disease resistance.

Preliminary results from these investigations have shown that peripartum cows can be divided into high and low immune response phenotypes. Low immune responsiveness during the peripartum period may relate to the physical and metabolic stresses encountered at calving, and likely contributes to the high incidence of disease during this period. It may be possible to readily identify high and low immune responder animals early in life using phenotypic and/or genetic markers, and to select cows with superior health characteristics prior to entry into the milking herd.



PHOTO: MASTERFILE, BARRETT & MACKAY

A similar strategy has already been developed for swine in which high and low immune response piglets are identified soon after weaning (see article on pg. 45). Identification and selection for high responders is based on estimated breeding values of immune response traits used in a multi-trait selection index developed by Bruce Wilkie, Bonnie Mallard and the late Brian Kennedy at Guelph. High immune response pigs are not only healthier, but they also exhibit superior performance characteristics, such as increased weight gain and litter size. This may relate to the fact that healthier animals need to divert less energy from production into health maintenance. This genetic approach to animal health maintenance also minimizes the need for antibiotic and chemical inputs, thus providing an environmental advantage. Development of this type of health and breeding scheme based on enhanced immune responsiveness is being extrapolated to other livestock, including cattle, keeping in mind the particular health requirements of that species.

Edible Vaccines

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Humans have always turned to plants for medicinal help in treating ailments and preventing disease. In a novel project inspired by researchers in the U.S. who are trying to develop 'edible vaccines' for humans using plants, our lab has teamed up with researchers Eva Nagy (Ontario Veterinary College) and Stephanie DeGrandis and Arlene Yee (Laboratory Services, OMAFRA) to genetically modify alfalfa to create an edible vaccine for swine. Our hypothesis is that pregnant sows will develop immunity to the transmissible gastroenteritis virus (TGEV), when they eat the transformed alfalfa as a component of their rations.

TGEV is an intestinal pathogen which causes acute diarrhea in pigs of all ages, and results in high mortality in piglets under 10 days of age. The virus is acquired orally and survives passage through the gut to the small intestine where it invades and destroys the absorptive tissues. Pigs which survive can regenerate this tissue and develop what is known as mucosal immunity, characterized by secretion of IgA antibodies into the intestinal cavity. The presence of these antibodies outside the cell prevents the binding and invasion of the virus particle, and hence an exposed pig becomes immune to the disease.

A feature of this mucosal immunity is the associated secretion of IgA into the sow's milk, conferring passive immunity to the suckling piglets, since the antibodies now perform the same function in the piglets' intestine as in the sow's intestine.



PHOTO: CARROLL AND CARROLL

Fig. 2. Sows exposed to transmissable gastroenteritis develop mucosal immunity and secrete IgA into their milk, conferring passive immunity to their suckling piglets.

TGEV is a well-characterized coronavirus with numerous club-shaped spikes extending from a membranous envelope (Fig. 1). The spikes, composed of a protein coded for by a viral gene, are used by the virus to attach to and invade the cells lining the intestine. The spike (S) protein is also the antigenic agent which triggers the immune response in the pig.

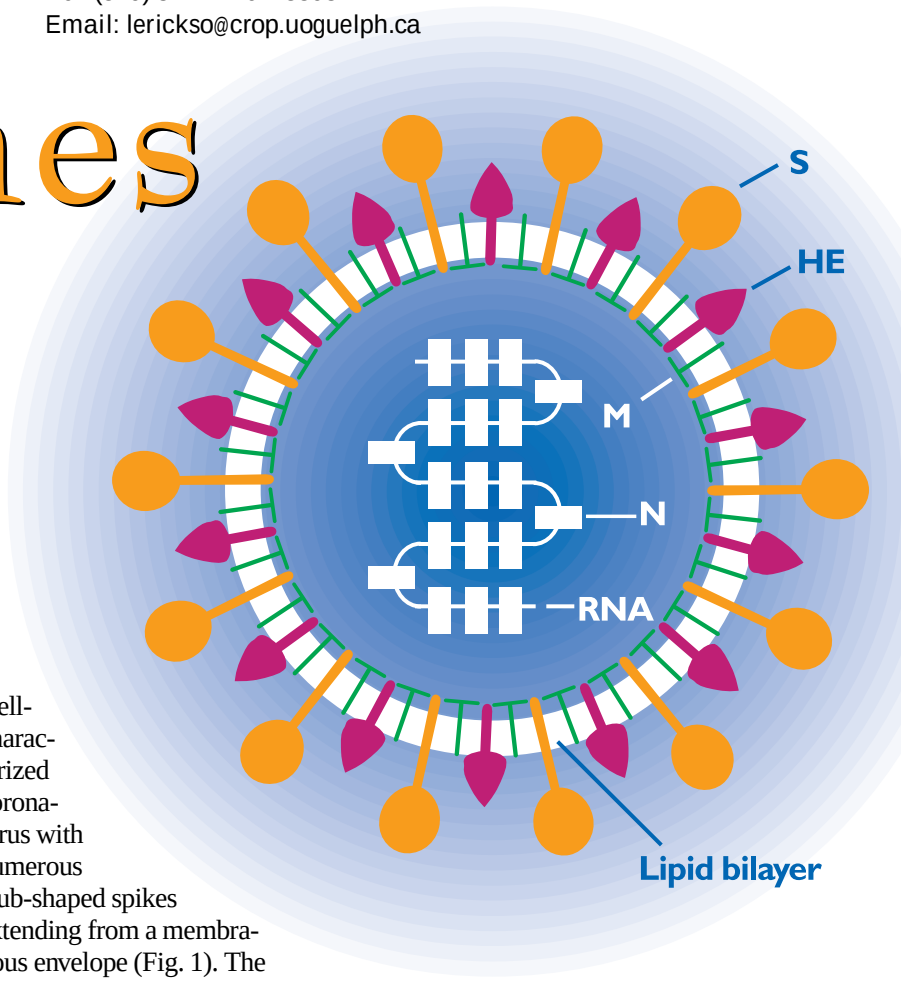


Fig. 1. The coronaviron structure of the transmissable gastroenteritis virus. Spike protein (S), hemaglutinin (HE), matrix protein (M), nucleocapsid protein (N), lipid bilayer, and genome (RNA) are shown. The HE protein is present in some, but not all coronavirus species.

The S protein gene has been cloned and characterized by Nagy and associates in the Ontario Veterinary College. Expression of this gene in insect cells has shown that the protein can be produced in host organisms other than swine, and that the protein is sufficiently similar, such that it is recognized by the swine antibodies. We believe we can duplicate these results in alfalfa.

In our lab, we have taken the S gene and modified it so it will be expressed throughout the whole plant, or only in specific tissues. We have transferred the S gene to alfalfa and tobacco and will be conducting feeding trials by the end of the year. Collaboratively we will then determine how to produce the S protein in a particular tissue and sub-cellular location to optimize its delivery and immungenicity.

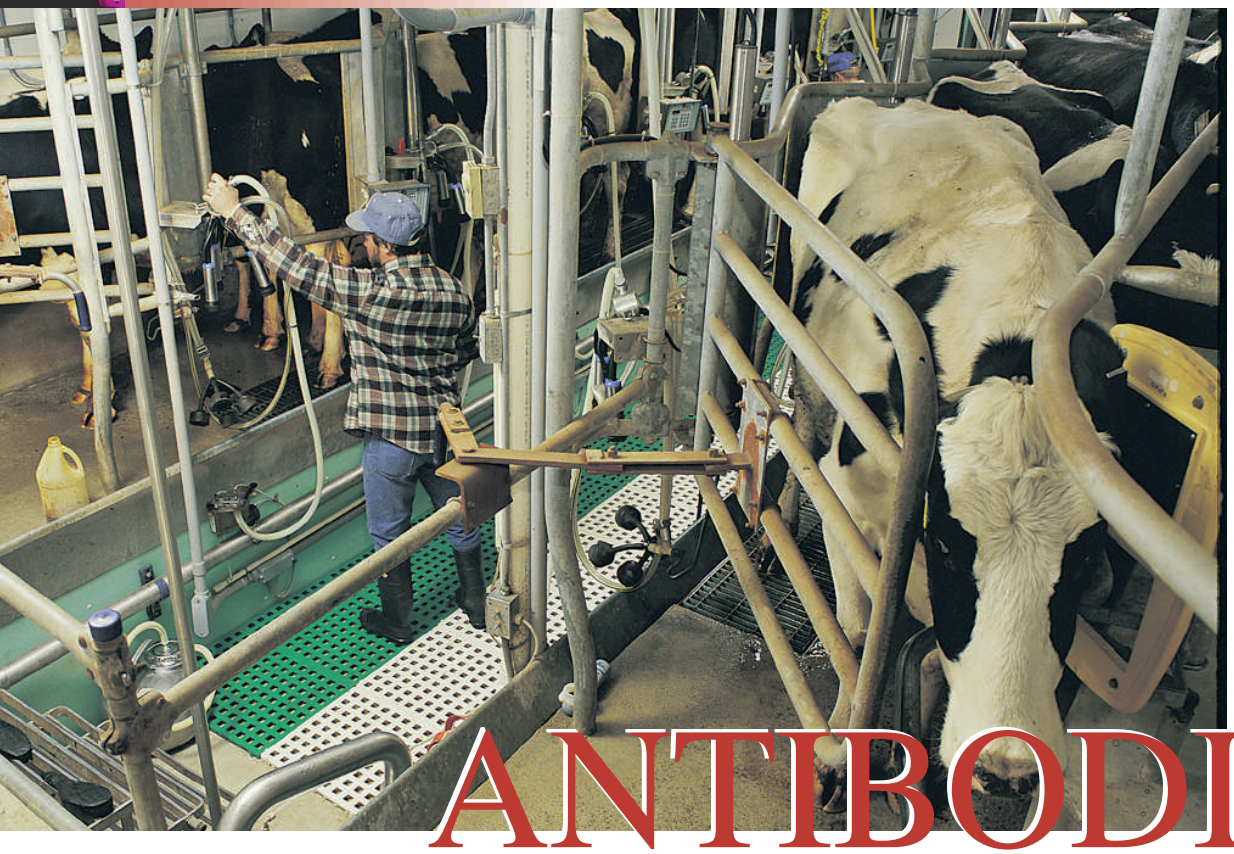


PHOTO: GRANT HEILMAN PHOTOGRAPHY

ANTIBODIES

that act as hormones

The antigen-binding region of antibody molecules can be arranged in an infinitely variable manner so that a configuration specific to any foreign substance can be created. In whole animal terms, this requires substantial gene rearrangements to generate polypeptide chains of the correct amino acid sequence. Another application that has been described is the production of antibodies with enzyme-like activity, so-called catalytic antibodies.

"Our approach has been to attempt to harness the power of this system to generate antibodies with a variable region that resembles a hormone – ligand antibodies – with potential for commercial application."

Long Acting Alternative to bST

We chose to study this approach using bovine somatotrophin (bST) as a model hormone with immediate commercial relevance, because its effect in promoting milk production in dairycattle is now well established. Our objective has been to create antibodies in lactating cows that mimic bST, with the prospect of increasing milk production.

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To accomplish this we used monoclonal antibodies raised against bST as antigens in dairy cows (Fig. 1), first raising a panel of monoclonal antibodies against bST in mice. Antibodies raised against bST possess a region which is a molecular mirror-image of a portion of the bST molecule. Using monoclonal technology, we can isolate antibodies specific to distinct regions of bST. If the anti-bST antibodies are then used to immunize cows, these animals will generate antibodies which mirror the anti-bST image of the antibodies. As the second mirror image, these should bear a strong resemblance to the region on the bST molecule that was originally recognized. If this is the region of bST that confers biological effects on cells, then this antibody should behave much like a supplement of exogenous bST.

Since antibodies have a longer half-life in circulation than bST, this would generate a long-acting mimic of bST in the circulation of the cow. With repeated vaccinations, therefore, this technique offers an opportunity for long term manipulation of milk production without the requirement to administer substantial quantities of a recombinantly produced hormone (rbST).

Defining the Antigenic Determinant Regions of bST

Using biological assays we determined that the monoclonals either inhibited the activity of bST (as would be expected) or, paradoxically, promoted the actions of bST in vivo. Monoclonals of both types were used as antigens in the lactation experiment. Immunization against the bST-inhibiting antibodies had no effect or marginally increased milk production (the latter is what we expected). Immunization against the bST-promoting antibodies actually decreased milk production – again this might be expected given the original activity of these antibodies.

We have, therefore, been successful in demonstrating that this approach can be used to alter physiological processes and performance, even though the results do not always confirm our predictions. The next step will be to more accurately define the antigenic determinant regions of bST and how these relate to the biological activity of bST and anti-bST antibodies.

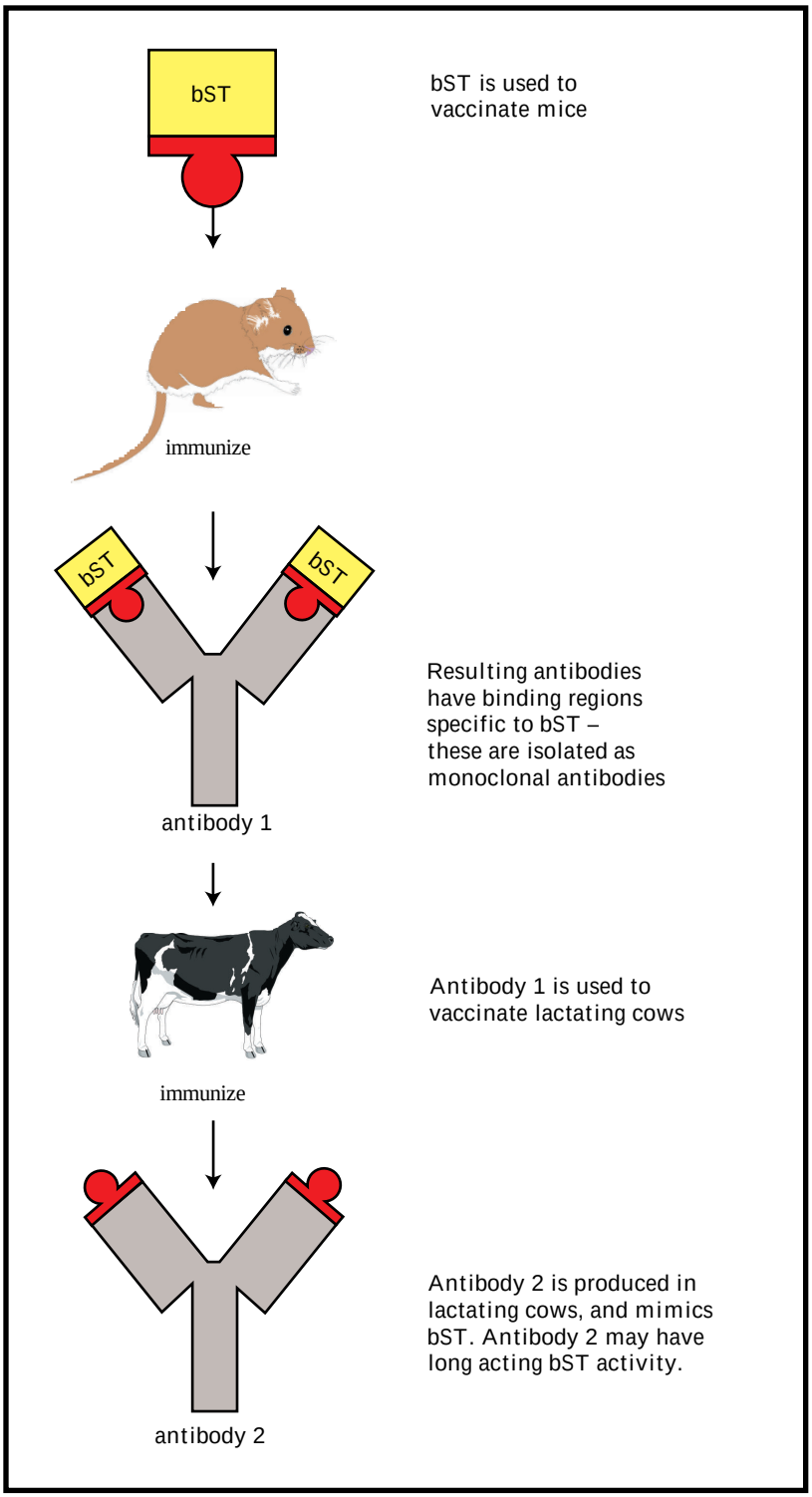


Fig. 1. Protocol to generate antibodies that mimic bST.

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Battling BOVINE LEUKEMIA VIRUS

Retroviruses are a unique group of infectious agents that are able to incorporate themselves into the host cell genetic material. This strategy allows for persistent infection of the host, since its defence mechanisms are largely ineffective. The three families of retroviruses, all of which are represented in cattle, are the oncornaviruses, lentiviruses, and spumaviruses.

The oncornavirus of cattle is the bovine leukemia virus (BLV); about 30% of dairy cows in Ontario are BLV-infected. The most important (albeit rare) disease caused by BLV is a malignant tumor of lymphocytes, termed lymphosarcoma. The lentivirus of cattle is the bovine immunodeficiency-like virus (BIV). BIV infects approximately 5% of dairy cows in Ontario and has been associated with decreased milk production. About half of Ontario's dairy cows are infected with a spumavirus called the bovine syncytial virus (BSV); there are no apparent disease associations with BSV.

Overall, many dairy cows are infected with retroviruses but the frequency of associated disease is low in comparison with the retroviruses of other species; for example, the feline leukemia virus (FeLV) and the human immunodeficiency virus (HIV). There is no evidence that the bovine retroviruses can infect humans.

Biggest Impact on Trade Barriers

Despite causing relatively little disease, the bovine retroviruses are economically important, since trade barriers exist against Canadian dairy cattle and bovine genetic material from BLV-infected farms or animals. Producers involved in the export business are most severely compromised. Currently, trade barriers are based on serological evidence for BLV infection. It is possible that in the future, trade barriers may be extended to include the other bovine retroviruses and that consumer acceptance of animal food products derived from retrovirus-infected cattle will decrease.

'Blocking Activity' May Inhibit BLV Replication

In order to develop strategies to limit transmission of BLV, a good understanding of the factors regulating expression of BLV is required. Under certain conditions, the retroviruses can produce additional virus particles while at other times the retrovirus remains quiescent, and is transferred to the infected cell's progeny only during normal cell replication. We have found that a soluble factor(s), termed 'blocking activity', in the blood of all cattle potently inhibits BLV replication. It appears that cattle produce the blocking activity continuously at low levels. Such a factor may have appeared in cattle as an adaptive strategy to deal with relatively frequent retroviral infections, hence, the low prevalence of disease associated with BLV.

Our main aims currently are to characterize the molecule(s) responsible for the blocking activity, determine if blocking activity influences the expression of the other bovine retroviruses, and to study the phenomenon across a variety of species.

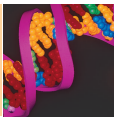
Future work will focus on understanding the modulators of the blocking activity. Host genetic characteristics, presence of other bacterial and viral infections, and environmental variables may be important in influencing blocking activity. We hypothesize that transient decreases in blocking activity allow for transmission and the replication of BLV. If this is correct, then it should be possible to design strategies to maintain BLV in a quiescent state. These, together with conventional infectious disease control methods should allow producers to decrease and eventually eliminate BLV infections in their herds over time.



PHOTO: CARROLL & CARROLL



Acknowledgement: This research is supported by NSERC, OMAFRA and the Dairy Farmers of Ontario.



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Biotech Food Safety Diagnostics:

Big Benefits Big Business



PHOTO: MASTERFILE: TED HOROWITZ. SALMONELLA BACTERIA

The use of diagnostics for agricultural and food safety has been making significant profits for manufacturers of immunoassay and molecular-based diagnostic kits. The current food safety testing business is already about $1 billion U.S. worldwide, and the market for rapid-testing procedures is growing around $150 million annually, with a projected growth to $400-500 million by the year 2005.

Test Centre for New Diagnostics

Scientists at GENEOLOGICS, a research service of the OMAFRA Agriculture and Food Laboratory Services Centre, are evaluating early stage (before market availability) rapid tests for several diagnostics manufacturers, to detect bacterial and parasitic contaminants of food and water such as *Salmonella*, *Listeria*, *E. coli* O157:H7 and *Cryptosporidium*. These organisms are considered major human health problems. All of the new and novel diagnostic tests under development are based on the ampl-ification of pathogen-specific DNA determinants using the polymerase chain reaction (PCR).

The practical problems that have prevented PCR from becoming a routine assay in agricultural and food safety labs have almost been resolved by the elimination of time-consuming steps after PCR, such as agarose gel detection of PCR products. Gel-based approaches have been replaced with ELISA immunoassays or closed-tube flourogenic product detection.

Highly Sensitive Detection

Using the DNA extraction techniques developed in our labs, as few as three to five *Salmonella* cells/25 g of food can be detected in naturally contaminated chicken carcasses, beef and milk samples, with the new fluorogenic 5'assay from Perkin-Elmer Applied Biosystems, California (Taqman LS-50B PCR detection system) and a 96 well format. The same technology allows the detection of *Listeria monocytogenes* (1000 CFU/25 g) in processed foods such as coleslaw. A similar flourogenic system developed by Biotronics of Lowell, MA called the Amplisensor system also allows homogeneous amplification and detection of PCR products. We have found that the assay is approximately two orders of magnitude more sensitive than detection by electrophoresis.

Our scientists, in collaboration with the ministries of Health, Energy and the Environment and the University of Guelph, have also developed a unique molecular-based ELISA assay for the detection of *Cryptosporidium* in Ontario's municipal waters. Using a novel amplification strategy we will be testing Ontario municipal waters for *Cryptosporidium* oocysts. This research is timely; Collingwood, Ontario experienced an outbreak of cryptosporidosis in March 1996.

In the next year, we will see PCR-based assays in the market which are highly automated; all functions including set-up and data analysis will be controlled by a single computer with an intuitive graphical user interface. Technicians will not require special training to run these assays. Both agricultural and food safety laboratories will benefit from the new and exciting PCR technology poised to enter the markets of the future.

A DNA Hybridization Method for Isolation of Verotoxigenic *E. coli* from meat products

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Strains of verotoxigenic Escherichia coli (VTEC) associated with foods of bovine origin have been implicated in outbreaks of hemorrhagic colitis and hemolytic uremic syndrome. These microflora of the gut of bovine animals can cause fatal disease in elderly, young and immunocompromised patients.

Isolating VTEC Strains from Mixed Bacterial Populations

The challenge in detecting these microorganisms by culture methods is the isolation of the strains from mixed bacterial populations present in food samples. A federal – provincial – University of Guelph collaborative team has developed a sensitive and specific DNA hybridization method to detect VTEC in meat samples, screened positive by a cytotoxicity Vero cell assay (VCA).

A DNA probe which was specific for verotoxin genes, was prepared from several well characterized VTEC strains and labelled with digoxigenin-dUTP by the polymerase chain reaction (PCR). The probe was hybridized with colonies immobilized and lysed on hydophobic grid membrane filters (HGMF) (Fig. 1). Positive colonies which hybridized with the probe were stained purple, and saved for strain characterization and serotype determination (Fig. 2).

Hybridization Method Recovered VTEC From More Samples

In 1995, over 1,000 meat samples, including 327 raw beef and pork and 744 processed ready-to-eat meat products were screened for toxin by the VCA. Filtrates of positive VCA samples at 1:10 dilution were filtered through an HGMF, and the HGMF was incubated overnight at 37°C. Filters with bacterial growth were replicated and prepared for hybridization with the probe. The samples were also tested by the conventional method of random picking and toxin testing of individual colonies.

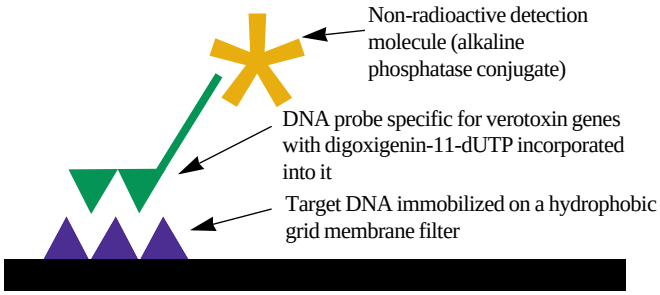


Fig. 1. A non-radioactive verotoxin-specific DNA probe hybridized with verotoxin-producing *E. coli*, immobilized on hydrophobic grid membrane filters.

The DNA hybridization method recovered VTEC from five additional samples that remained undetected by the conventional method. The minimum level of detection was determined to be as few as 1.5 cfu per 25 g of ground beef. Colonies which were isolated were confirmed as VTEC by biochemical testing. We were able to perform characterization tests of the strains for toxin type, serotype, adherence factors, hemolysin genes and the presence of plasmids.

Ideal for Food Testing Labs

Whereas other commercially available rapid probe-based methods for detecting VTEC still rely on conventional culture and isolation methods to recover the organism from the sample, this method combines detection and isolation procedures. Once samples are screened positive by a screening test (in this case the VCA), VTEC strains are successfully recovered from these samples using our probe method. In this respect, our method is more efficient over the random testing of colonies.

Although we have successfully applied this method to meat samples naturally contaminated with VTEC, we believe it can also be applied to other types of food for the recovery of other food-borne pathogens,using appropriate oligonucleotide probes to target known virulence genes in the bacteria. The fact that this method does not require radioactive labels makes it ideal for use in food testing laboratories.

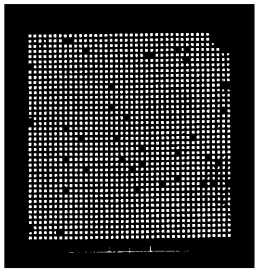


Fig. 2. Purple stained isolated verotoxin-producing colonies on a hydrophobic grid membrane filter. Positive control H30 strain in top right-corner. Negative *E. coli* ATCC 25922 in top left corner.



Toward Rapid On-Line Detection of Pathogens *for food-processing*

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While Canada enjoys one of the safest food supplies in the world, an estimated two million cases of foodborne illness occur here each year. Many could easily be prevented with more careful food handling practices by consumers. As well as the unmeasured social cost, there is an economic toll associated with medical care and lost work time amounting to millions of dollars.

Althought the vast majority of food we eat is perfectly safe, there is a need to maintain vigilance to ensure that food safety is not compromised. This is particularly important as new food processing technologies are constantly being developed, 'new' foodborne pathogens emerge, and the number of seniors in our society increase.

Government and Ontario industries are taking steps to further improve the high standard of our food supply, such as implementation of Hazard Analysis of Critical Control Points (HACCP for short). This procedure requires identification of the stages in food processing that are most critical to avoid contamination by harmful bacteria. To verify HACCP is working, microbiological tests are needed that identify potential problems quickly and reliably. My research group is using the tools of biotechnology to give the industry the methods it needs to perform this task.

The polymerase chain reaction (PCR) is a method for amplifying a segment of DNA that is unique to a particular organism. Copies of the original sequence can be detected in a variety of ways, but we are using a method that produces light if the target segment, and hence the organism, is present.

To do this, we label a complementary strand of the DNA sequence with a compound called digoxigenin. The labelled sequence binds (hybridizes) to the amplified target, and an antibody to the digoxigenin with an enzyme attached is then added. A chemiluminescent substrate allows detection of the hybridized

product by light emission. Sensitive instrumentation means that we can pick up small amounts of light and therefore detect low numbers of the organism of concern in the original sample. The application of PCR to foods is in its infancy but we have developed methods of superior sensitivity that are capable of detecting less than one cell of a pathogen per ml of milk, for example.

Although substantial advances in PCR methodology are being made, it is still not a method that can be used routinely by workers on the factory floor. More robust and convenient methods are required for that application.

Phages Reveal Pathogenic Hosts

Toward that end, work in my labs is aimed at using bacteriophages to detect potential pathogens in food. Bacteriophages (or phages) are viruses that are specific to certain types of bacteria. It is possible to identify a phage that will only attack *Salmonella*, for instance. By determining if the bacterium has been attacked by the phage, we can tell if the food is contaminated. We are investigating several ways to detect phage infection of the host cell.

One method is to introduce the genes responsible for bioluminescence (the ability to emit light) into the phage (Fig. 1). Light is only given off when the phage attacks its host. By the use of sensitive light detection and image analysis instrumentation, it is possible to determine the presence of an undesirable organism directly in foods (Vol. 16 No. 3). A method has been developed that allows the detection of *Salmonella* directly in whole eggs within 24 hours.

Phage-based detection systems are becoming commercially available, and they offer an inexpensive, easy to use method to help ensure food safety.

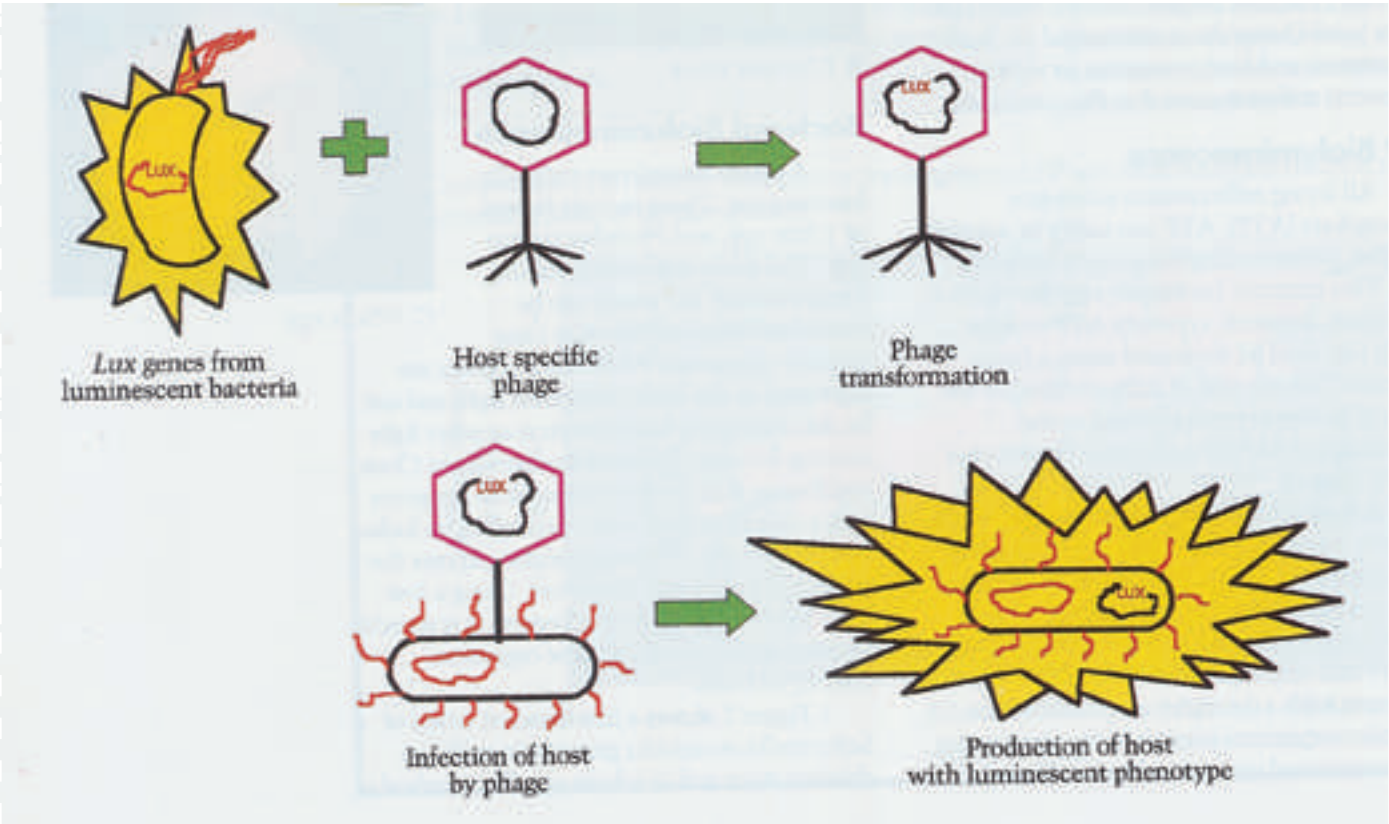


Fig. 1. Schematic representation of bacterial detection in food, using genetically modified bacteriophage.

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Scientists at the OMAFRA Agriculture and Food Laboratory Services Centre in Guelph are studying the application of systems for identification and epidemiological typing of food-borne pathogens, isolated from a variety of food samples. One system being examined is the fatty acid-based *Microbial Identification System* (MIS), which employs computerized high-resolution gas-liquid chromatography to determine the fatty acid (FA) composition of a bacterial isolate. FA methyl esters, dimethyl acetyls, aldehydes and unknown compounds unique to a bacterial species are identified by the system. The FA composition of the unknown isolate is then compared by the system to that of a reference database of the cellular FA profiles for more than 1,800 bacterial species.

In a recent federal-provincial-University of Guelph collaborative project, a library of the FAs from more than 600 bacterial isolates of Salmonella enteritidis, Listeria monocytogenes, Verotoxigenic E.coli and Bacillus species was produced. Attempts were made to group bacterial species based on their fatty acid composition. The system was found to be useful in grouping dairy isolates of Bacillus species, but appeared limited for the other genera. The use of the system for developing a fatty acid-based typing scheme may work for some groups of organisms and not others. Further studies on the application of the system for tracking other bacterial species associated with food-borne illness are in progress.

Epidemiologic

Typing of Food-borne Bacteria

The MIS results of the FA analyses include the names of the FA derivatives, their retention times and amounts, and a list of probable identifications with similarity index (SI) values. The SI represents the closeness of the FA profile of an unknown isolate to representative profiles in the database. FA profiles are compared statistically using pattern recognition techniques. Figure 1 shows a typical FA profile of bacteria as it appears on the MIS print out. The MIS is fully automated with the capability of processing more than 80 bacterial FA extracts per day unattended, at a cost of $2.50 Canadian per sample. Although designed primarily for bacterial species identification, it has the potential to be discriminatory at the bacterial strain level, and to group like organisms in clusters - useful for epidemiological purposes in tracking the source(s) of food-borne illness outbreaks.

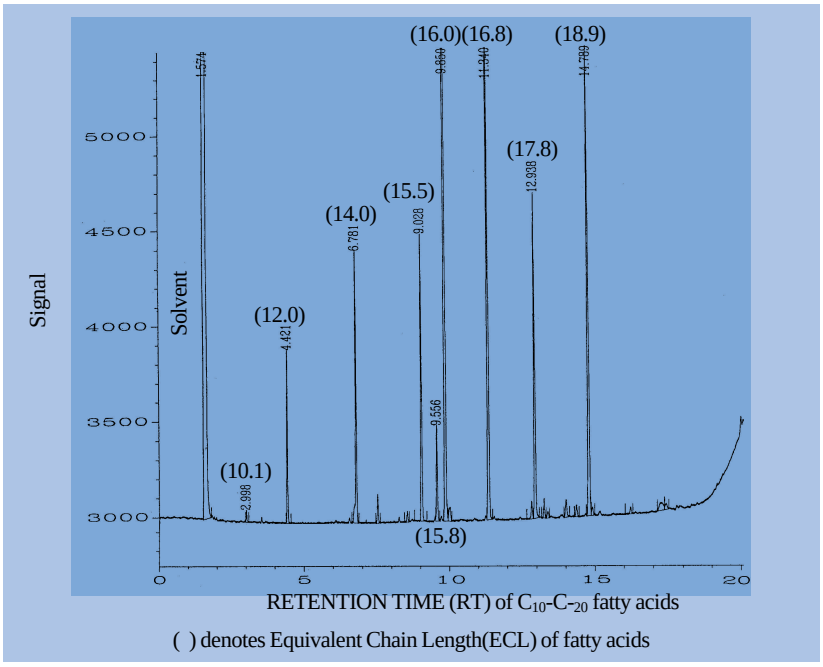


Fig. 1. Fatty acid profile of *E. coli* 0157:H7 (ATCC Strain No. 43895)

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BIOTECHNOLOGY IN Food Processing: *taste and quality top the list*

There's a tractor-trailer rolling down Highway 401, brimming with horse radish grown in the Brantford area. Its destination: a bioprocessing plant in Winnipeg, where highly skilled technicians will turn the multi-tonne load into just two kg of a highly-purified enzyme called peroxidase, the active ingredient in many bioassay tests that directly measure, among other things, blood glucose and blood cholesterol. The processing extends horse radish from the dinner table to the big leagues: two kg of peroxidase is worth a whopping $150,000.



PHOTO: FOODLAND ONTARIO

That's only one of the many opportunities biotechnology offers the food industry. Besides the creation of the bioassay itself, there's potential for biotechnology to add even more value to the raw product. For example, why not genetically modify the horse radish to have twice as much peroxidase as it has now? Or engineer the plant so it also produces other beneficial enzymes that can be harvested and extracted at the same time?

The Guelph Food Technology Centre (GFTC) makes connections between researchers and processors. It's a one-stop shopping location for entrepreneurs who want to develop new products, improve product quality, enhance productivity through the use of new technology or strengthen its employees' skills.

By making this connection, the GFTC is helping integrate biotechnology into the industry. In its first calendar year, the centre had 150 entrepreneurs or projects come through its doors. The GFTC matched them with research expertise, most of which is at the centre itself or the University of Guelph. This connection helps researchers focus their activity on the end user – the consumer.

Foods with a Competitive Edge

Biotechnology can make food more user-friendly for consumers. A good example is the bioengineered enzymes now on the market to increase the digestibility of food like milk and beans. Without the enzymes, the foods have limited usefulness to many people. With them, consumers have more choices and industry has more customers... and incentive to expand into other novel product areas using the latest technologies.

In 1995, a record 23,000 new food products hit North American store shelves, up 12 per cent from the previous year...and 1996 is tracking even higher.

Traditionally, a large percentage of new products have failed. The tools of biotechnology can now give them a competitive edge — through safer, more efficient processing, improved nutrition, or new uses for existing products. Will industry buy into it? That load of horse radish barreling down the 401 says it all.



PHOTO: MASTERFILE, KEN DAVIES

Genetically Engineered Wine Yeasts for Cool Climates

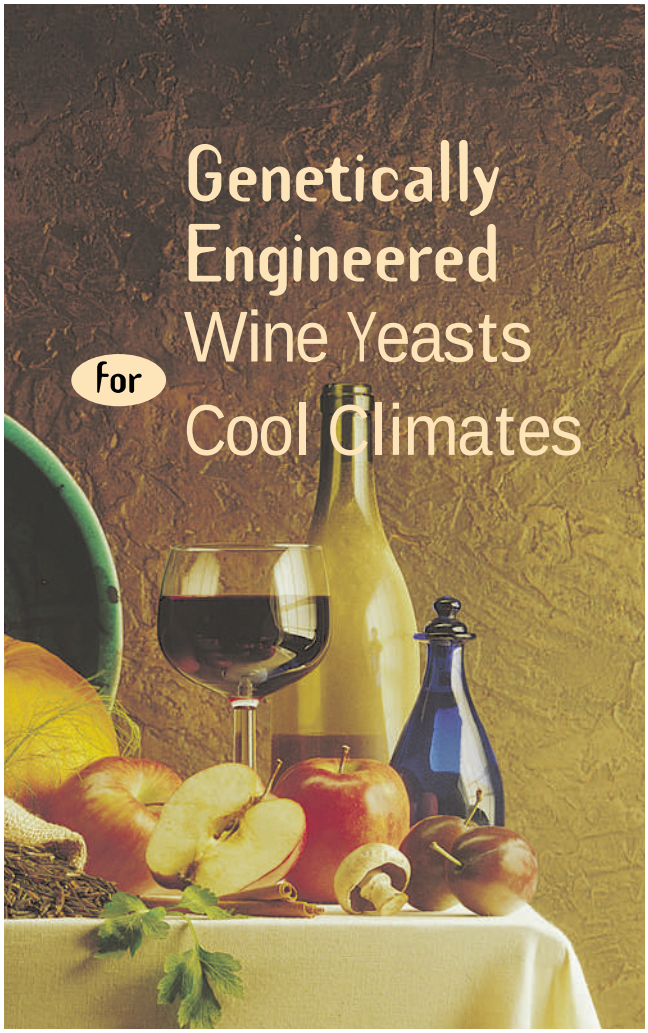


PHOTO: CARROLL & CARROLL

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One of the challenges of wine-making in the cooler latitudes is the extremes of summer weather, affecting the desired ripeness of the grapes. The grower measures ripeness in terms of sugar, acid and flavour. As the grape matures, the sugar increases, the acid drops and the flavour increases, then decreases. Due to the shorter growing season in cooler climates, grapes are often harvested earlier, resulting in a higher than desired acid content.

Traditional Treatment of Excess Acidity

The principle acids in grape juice are malic acid and tartaric acid. Wine-makers like to adjust acidity by dealing with the malic acid, since it has a more sour taste than tartaric acid. Malic acid can also support the growth of lactic acid-producing bacteria that spoil a wine after it has been bottled.

Traditionally, vintners have dealt with excess acidity by one of four methods: 1) dilution: sugar and water are added to the grape juice to dilute out the malic acid. 2) titration: powdered chalk is added to titrate the acids. 3) edulcoration: sugar or grape juice is added to the finished wine, or 4) malo-lactic fermentation: certain lactic acid-producing bacteria can change malic acid into lactic acid, a much mellower tasting acid.

The first three methods all leave residual malic acid in the wine, so a preservative like sulphur dioxide is added to inhibit bacteria and stabilize the wine.

Malo-lactic fermentation involves bacteria which, in addition to converting malic acid into lactic acid, also degrade many terpenes and other molecules that contribute to the fruity and floral character of Riesling and Gewurztraminer.

Through the 1980s we worked to construct a wine yeast that could ferment malic acid. After conventional hybridization methods using *Schizosaccharomyces pombe* failed, we decided to clone the genes responsible for malic acid fermentation and move them into the wine yeast Saccaromyces cerevisiae.

By 1986, we had identified the genes responsible for malic acid fermentation and three years later we cloned these genes. The genes coded for a malic acid transport protein (not found in the genome of the wine yeast) and a very active malic enzyme. Whereas malic acid entered *S. cerevisiae* by facilitated diffusion and entered into an incomplete citric acid cycle, the *Sch. pombe* had malic acid actively transported into the cell and converted quickly to pyruvate, which was rapidly converted into ethanol. The differences between the two species were quite striking. Unfortunately, we could not get the wine yeast to recognize and express either of the genes.

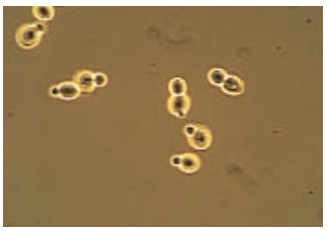
We subsequently entered into a collaboration with H.J.J van Vuuren of the University of Stellenbosch in South Africa. He sequenced the genes and used a number of expression cassettes until he finally expressed the pathway in *S. cerevisiae*.

Another collaborator, Aline Lovaund of the University of Bordeaux, France, had successfully cloned the gene to convert malic acid into lactic acid. The malate transport gene was combined with the malo-lactic gene.

Collectively we have produced the first yeast strain that will perform a malo-ethanolic fermentation (desirable for Riesling, Gewurztraminer, Muscat and other fruity wines), and another that will carry out a malo-lactic fermentation (for Chardonnays and red wines). We are now back-crossing the recombinant strains to selected wine yeast to improve fermentation and organoleptic performance.

Use in Commercial Wine Production

We are presently patenting the organism and entering into negotiation with the industry for use of the organism in commercial wine production. For the vintner it will mean that the wines have finished all but aging and fining two weeks after harvest. Consumers will benefit from more consistent quality and less sulfite required in their wine.



Saccaromyces cerevisiae **x 400.**

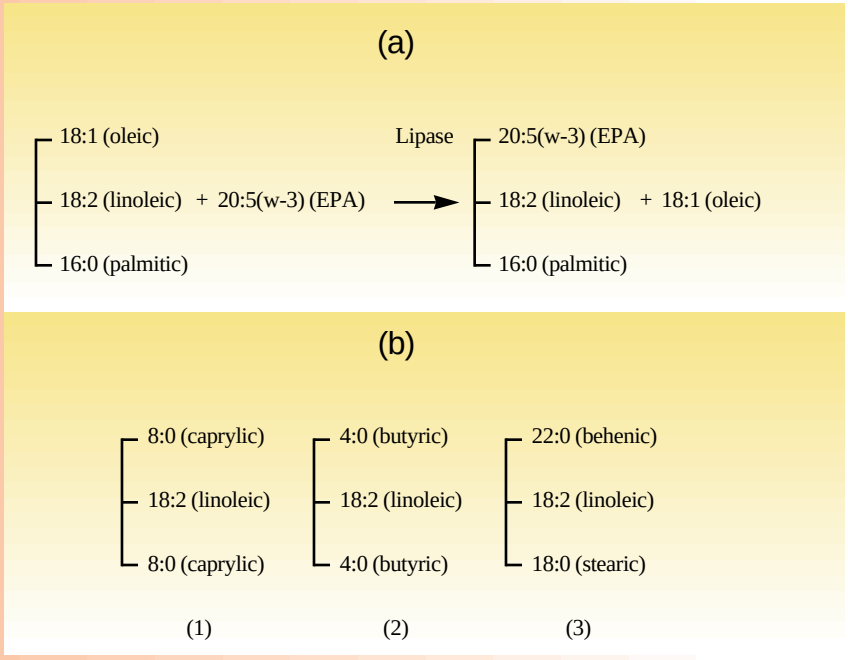


PHOTO: MASTERFILE, MARK TOMALTY

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'Designer' FATS & OILS



Enzymes, powerful biological catalysts, have played a key role in the development of biotechnological industrial processes. Advantages of enzymes over conventional chemical catalysts include substrate specificity, lower energy costs and a higher quality end-product, not to mention the "natural", "no chemicals added" image.

Fig. 1. a) Incorporation of EPA (eicosopentaenoic acid, a fish oil) into a putative vegetable lipid by enzymatic interesterification. b) Three examples of triglycerides that could be synthesized by enzymatic interesterification with great economic potential for food and pharmaceutical industries.

The use of enzymes for the production of popular food products such as cheese and wine probably dates back several millennia. One of the earliest recorded patents for the use of enzymes dates back to 1894 when a process for making diastatic enzymes (amylases) used in the brewing industries was patented. Since then, the use of enzymes in manufacturing has exploded, particularly with the growth of molecular biology. The great bulk of enzymes used in industry are microbial in origin, mainly due to production costs and the potential for genetic modification.

Improved Biological Activity, Physical Properties and Sensory Characteristics

Our research in the past few years has concentrated on the use of lipases for the manufacture of modified lipids (fats and oils) for the food and pharmaceutical industries. In excess water, lipases hydrolyze ester bonds in triacylglycerols; however, under water-limiting conditions (microaqueous), lipases will also resynthesize the ester bond. This offers the potential to incorporate foreign fatty acids into a particular triglyceride, essentially tailor-making a particular molecular structure. For example, potential applications could include modifying the spreadability of a fat, manufacturing a fat that will combat cardiovascular disease, manufacturing a hypocaloric fat – the imagination is the limit.

Figure 1 shows a putative acidolysis reaction (triglyceride with an added fatty acid) between a generic vegetable fat triglyceride and eicosopentaenoic acid (EPA), a fish oil-derived fatty acid known to be beneficial to cardiovascular health. In that figure, we also show other modified fats with interesting properties. Medium chain fatty acids could be incorporated into the vegetable fat (1). These fatty acids do not raise serum cholesterol levels. Butyric acid-containing triglycerides (2) could be beneficial against colon cancer, while very-long-chain and long-chain fatty acid containing triglycerides (3) are not readily metabolized, hence provide less calories per gram than regular fats.

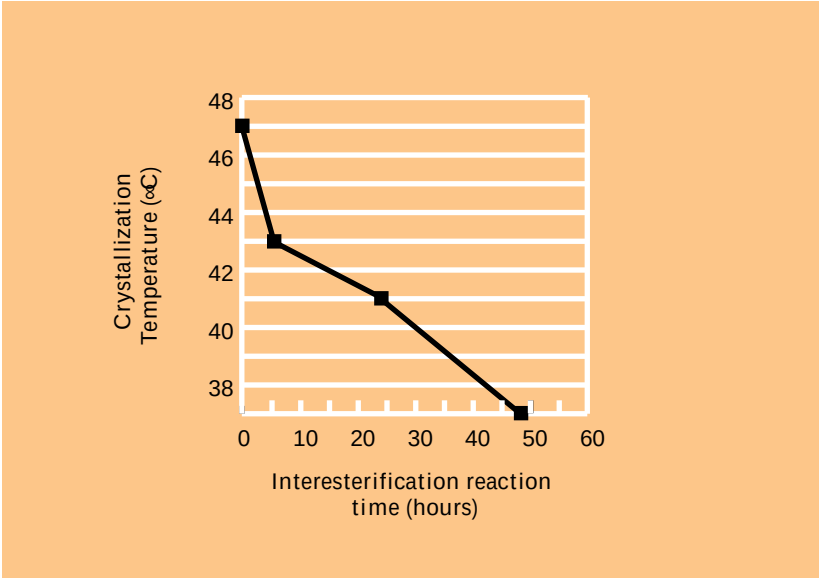
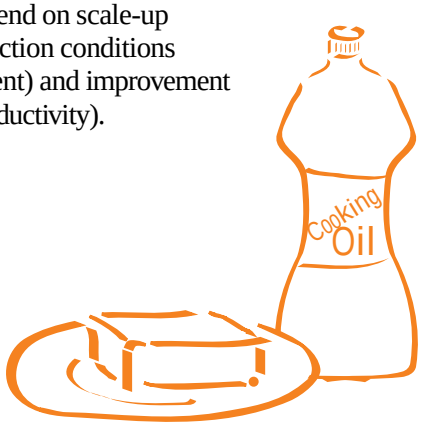


Fig. 2. Changes in the crystallization behaviour of a tripalmitin-triolein mixture as a function of enzymatic transesterification time. The crystallization temperature (midpoint of the curve) decreases as interesterification progresses.

Figure 2 shows results from an enzymatic interesterification between triolein (an oil) and tripalmitin (a hard stock). Notice how after 48 hours, the crystallization temperature of the fat (33% tripalmitin, 67% triolein) dropped from 47.7 to 37.5°C. This reaction produced a fat with improved physical properties, a lower melting temperature and a decreased solid-fat-content at room temperature.

Widespread application of lipase-mediated processes will depend on scale-up potential, control of reaction conditions particularly water content) and improvement of reaction kinetics (productivity).



Since the industrial revolution, human activities have resulted in the release of an enormous volume of toxic chemicals into the environment. Bioremediation is an attractive and ecologically sound method of removing many environmental contaminants, by making use of microorganisms to degrade pollutants to harmless substances. Alternatively, microbial enzymes may be used to transform the pollutants to intermediates which can then be broken down more readily by microorganisms.

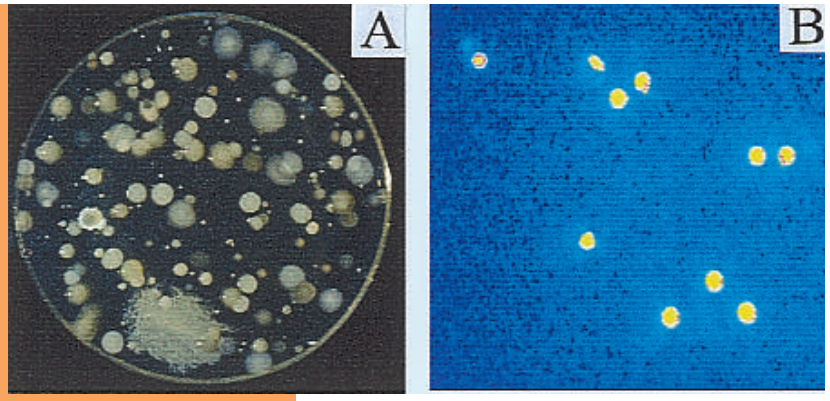


Fig. 1. Panel A: Microbial growth on a nutrient agar plate containing the genetically modified *Pseudomonas aeruginosa* UG2Lr and endogenous soil microorganisms. Panel B: Computer generated photo image of the bioluminescent UG2Lr cells from the same plate.

Cleaning up Contaminants with Customized Microbes and Enzymes

Indigenous Microbes May Lack Degradative Pathways

Despite the metabolic versatility and diversity of microorganisms, existing microbial degradative pathways may be inadequate to handle all previous and new pollutants being generated by industrial activities, since new degradative pathways require a long period of time to evolve. One approach to deal with persistent compounds is to genetically design microorganisms or improve catabolic enzymes to increase effectiveness of *in situ* bioremediation. Genetically marked microorganisms can also be used to construct model systems for improving bioremediation.

Research has been carried out successfully to increase the biodegradative ability of some microbes by introducing catabolic genes from good pollutant degraders into more robust microorganisms. For example, *Pseudomonas* sp. B13 is able to degrade 3-chlorobenzoate (3CB) but not 4-chlobenzoate (4CB). This strain was genetically engineered to express the 4CB-degrading toluate dioxygenase from *P. putida* strain KT2440 (TOL). The resultant strain was able to degrade both 3CB and 4CB. Eventually, catabolic genes responsible for complete degradation of some persistent compounds will have to be transferred to organisms that are competitive and better adapted to the toxic environment.

Assessment Prior to Environmental Release

Thus far, genetically engineered microorganisms (GEMs) have been used to remediate contaminated soils in contained environments such as laboratory microcosms. In future, GEMs may be released into the environment to degrade persistent pollutants. A major consideration with the release of GEMs is their potential risks to human and environmental health. To address these concerns, studies on the probability of survival, dispersal, colonization and function of released organisms and their introduced DNA in natural ecological systems must be carried out. To evaluate microbe-mediated ecological processes, marker systems are necessary to track and monitor GEMs in the environment

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In our laboratories, two marker systems, the luciferase (*luxAB*) gene from the bacterium *Vibrio harveyi*, and the green fluorescent protein (*gfp*) gene from jelly fish, are being used to monitor bacteria with good biodegradative potential. *Pseudomonas* sp. UG14, a phenanthrene-mineralizing bacterium, and *P. aeruginosa* UG2Lr, a biosurfactant-producing bacterium, both isolated from contaminated soils, were marked with *luxAB* genes. The bioluminescence in the GEMs is measured by either a luminometer or charge-coupled camera (Fig. 1). When introduced into creosote-contaminated soil, the *lux*-marked *Pseudomonas* sp. UG14 degraded phenanthrene and the fate of the bacteria was easily followed by the *lux*-marker system. The GFP, which absorbs blue light at 395 nm and emits green light at 509 nm, has been used as a marker system in different organisms. Green fluorescence in the GEMs is detected by fluorometry or by green light emitted.

Increasing the Range in which Enzymes Function

The actual task of degrading pollutants is done by enzymes produced by the microbes. Degradation may be inefficient, not only because of insufficiently evolved enzymatic pathways, but also because of enzyme instability under prevailing environmental conditions in the contaminated sites, or because the pollutants are simply too toxic. The biodegradative enzymes may also have a limited range of pH and temperature in which they function; outside this range they will be denatured.

One solution to these problems is to modify the enzymes either rationally or randomly so that they can function efficiently under adverse environmental conditions. This can be done by changing the gene which encodes the enzyme of interest. Altering the gene sequence changes the amino acids present in the enzyme. By using site-directed mutagenesis, precise substitution, deletion or insertion in any region of a cloned gene can be made to alter the amino acid sequence of the biodegradative enzyme in a pre-determined way. This may increase the pH or temperature range in which the enzyme functions or it can change the size and shape of the substrate binding pocket, thereby allowing new substrates to be catalyzed.

The approach may also allow the modified enzyme to function under harsh conditions. In our laboratories, we have successfully replaced the amino acid aspartate at position 170 with alanine in the enzyme haloalkane dehalogenase, which dechlorinates chlorinated aliphatic compounds. The modified enzyme can dechlorinate the substrate 1,5-dichloroethane, which the wild type enzyme can not. Successfully modified enzymes may be used in immobilized form to transform specific pollutants, or the gene encoding the modified enzyme can be introduced into a competitive microorganism. It can then be inoculated into contaminated sites to degrade the target pollutants.

The role of bioremediation in dealing with highly contaminated sites is in the early stages of development. As microbial products and regulatory requirements become harmonized, it may be possible to use genetically engineered microorganisms to bioremediate contaminated sites containing diverse range of environmental pollutants. It is important to note that in some contaminated sites, manipulation of physical and chemical conditions is sufficient for naturally occurring microorganisms to efficiently degrade the chemical contaminants. The optimal condition for biodegradation is a complex inter-relationship between the pollutant, the environment and the microbes (Fig. 2).

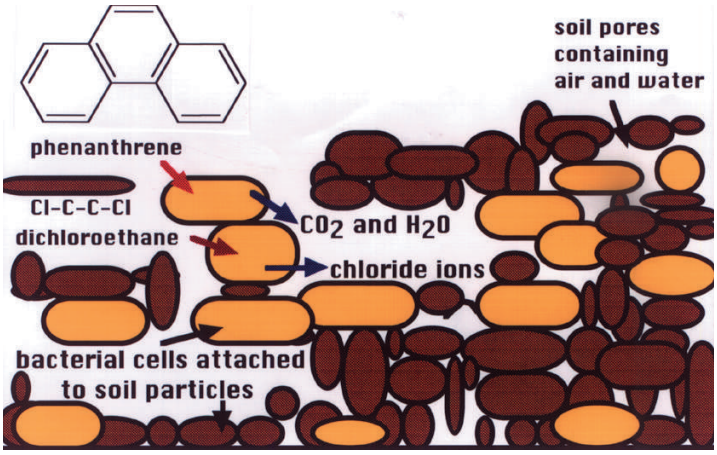
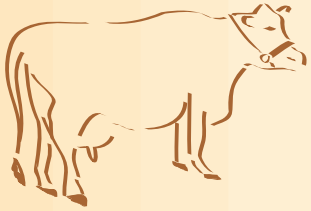


Fig. 2. Pollutants such as phenanthrene and dichloroethane can be transported in soil water and also complexed to soil particles.

AND MORE... Biotechno



PROGRAM/PROJECT*	RESEARCHER*
AQUACULTURE	
Molecular genetic analysis of cultured rainbow trout in Ontario	Moira Ferguson
Improved reproductive performance and cryogenic storage of gametes in salmonid aquaculture	Richard Moccia
Application of antibodies to surface antigens of fish immune cells in assays to assess fish health and immune responses	Roselynn Stevenson
BEEF	
Antibody sexing of embryos and sperm	Stan Blecher
Stress proteins, autoimmunity and autoimmune disease	Azad Kaushik
The effect of chromosomal composition and environmental stress on bovine embryo development and survival in vitro	W. Allan King
Characterization of antigens of *Pasteurella haemolytica* and their use in the development of vaccines against bovine pasteurellosis	Alan Mellors
Molecular analysis of extracellular matrix receptors (integrins) in bovine embryos	Alan Wildeman
DAIRY	
The production of identical calves from isolated blastomeres, fetal germ cells or embryonic stem cells	Keith Betteridge
Development of feed enzymes for enhanced forage digestion in ruminants	Cecil Forsberg
Development of a gene transfer system for *Fibrobacter succinogenes*	Cecil Forsberg
An accurate method of selecting dairy sires as calves utilizing the BST index	Roger Hacker
The use of follicular aspiration and *in vitro* fertilization to produce embryos from pregnant cows	Walter Johnson
Comparative molecular analysis of immunoglobulin genes and construction of the immune system	Azad Kaushik
Bovine lipoprotein receptors in mammary lipid metabolism	Jonathon Lamarre
EQUINE	
Factors influencing capacitating ability of equine epididymal spermatozoa and fertilizing ability	Mary Buhr
Rapid detection of *Salmonella* in the horse	Carlton Giles
Cryobiology of equine spermatozoa	Claire Plante
Immunity to *Rhoddococcus equi*	John Prescott
POULTRY	
Live avirulent vaccine against *E. coli* infections in broilers	Carlton Gyles
Parthenogenesis in turkeys	Rob Etches
dsRNA viruses of Eimeria species of the chicken: effect on the pathogenicity, immunogenicity and drug resistance of virus-infected Eimeria	Aggie Fernando

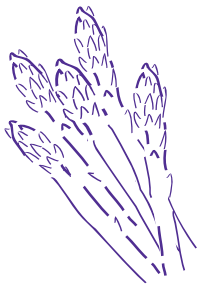




logy at Work in Research







PROGRAM/PROJECT*	RESEARCHER*
POULTRY Continued . . .	
Drug resistance in Eimerian parasites of the domestic fowl: the molecular basis	Aggie Fernando
Investigation of the molecular biology of avian viruses and development of recombinant vaccines and diagnostic procedures	Eva Nagy
SHEEP	
Improvements in the application of embryo and semen technologies to improve sheep production	Brian Buckrell
Towards the *in vitro* production of viable sheep embryos of known sex	W. Allan King
SWINE	
Strategies for genetic improvement in swine	John Gibson
Adaptive defences to environmental stress as molecular biomarkers of swine health	Kim Kirby
Molecular analysis of *Actinobacillus pleuropneumoniae* virulence factors	Jan MacInnes
Analysis of bacterial pathogens in Ontario swine	Jan MacInnes
Application of transgenic methodology for enhanced dietary efficiency and reduced waste pollution characteristics in swine	John Phillips
Development and clinical application of reproductive biotechnologies in swine	Claire Plante
FOOD	
The development and clinical validation of desginer foods with natural ingredients for preventing/managing cardiovascular disease	Bruce Holub
Technology development for the commercial isolation and purification yeast beta-galactosidase	Yukio Kakuda
Improving structure and properties of fruit and vegetable-derived processed foods by regulating the activities of undesirable and desirable reactions	Gopi Paliyath
Active packaging to control microbial growth utilizing naturally occurring products	Marvin Tung
Factors regulating the processability of Ontario grown potatoes	Rick Yada
CROP IMPROVEMENT & PROTECTION	
Development of transgenic tobacco plants resistant to nematodes	Barry Shelp
Generation of an alternate reference map for positional cloning of pest resistance genes in solanaceous crops	Judy Strommer
Development of improved asparagus germplasm	Dave Wolyn
Development of a rapid detection technique for grapevine corky bark and leafroll disease	Annette Nassuth
Development of ultra-sensitive methods for the detection of new low-use-rate herbicides in the environment	Chris Hall

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