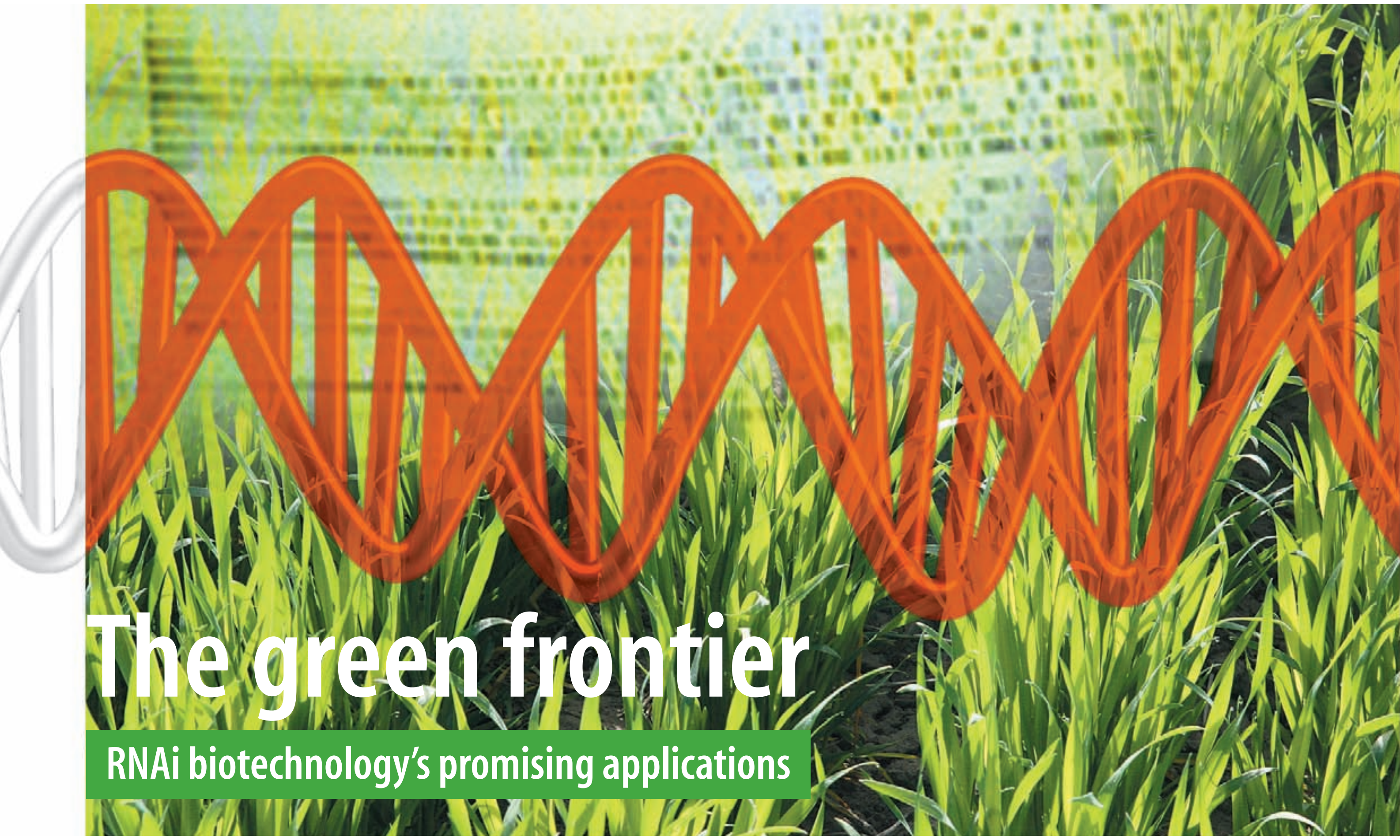


The green frontier

RNAi biotechnology's promising applications

A new research tool for molecular genetics, hailed as the most important technology since the polymerase chain reaction in the early 1980s, is transforming research across the medical and biological sciences. Agriculture, the environment and people will be major beneficiaries as RNAi provides researchers with non-chemical ways of controlling pests and disease, increasing yields and delivering essential foods. **Graeme O'Neill** reports.

At CSIRO Livestock Industries' annual *Horizons in Livestock Science* conference last September, Australian and international speakers described how RNA interference (RNAi) technology is being used to identify new molecular targets for drugs, to develop healthier foods, and to protect crops, livestock and aquaculture against diseases, pests and parasites. RNA is a nucleic acid that helps to transfer information from DNA to the protein-forming system of the cell. RNAi has emerged as a key technology for developing sustainable food production systems, and its advent could not be timelier.

In recent years, molecular geneticists have mapped, catalogued and sequenced the complete genetic blueprints of several dozen animals, plants and microbes. DNA sequences from hundreds of thousands of new genes have flooded international databases, giving researchers a glut of material to decipher.

The problem is that a gene's DNA code offers few clues to the function of the protein it dictates. The sheer volume of data has swamped the capacity of existing methods to determine what genes actually do in their host organisms.

But with RNAi technology, researchers can now selectively switch off (or 'silence') an anonymous gene in model organisms like the mouse (*Mus musculus*), the tiny weed, thale cress (*Arabidopsis thaliana*), the nematode worm (*Caenorhabditis elegans*), or brewer's yeast (*Saccharomyces cerevisiae*). The resulting changes provide strong clues to the silenced gene's function in the organism. It's a rapid way to decode genetic material.

A new European research project attests to RNAi's power to speed up the massive task of exploring the function of the myriad anonymous genes in plants, animals and microbes. The Agrikola research consortium aims to determine the function of all 20 000-odd genes in *Arabidopsis*, the model species for plant genetics.

In little more than six months, Agrikola researchers created more than 20 000 *Arabidopsis* lines (genetic types) to test, each with a different gene 'silenced' by RNAi. With previous, random gene 'knockout' techniques, it would have taken years.

CSIRO Plant Industry molecular geneticist Dr Peter Waterhouse told the *Horizons in Livestock Science* conference that the Agrikola project used an ingenious, plug-and-play 'gene cassette' called a 'Hellsgate' vector, developed by his colleague, Dr Chris Helliwell, which automatically creates a synthetic RNAi gene that, when delivered into *Arabidopsis*, will suppress the activity of a target gene. The resulting physical or biochemical changes in the plant offer clues to the gene's function.

CSIRO Livestock Industries, Plant Industry and Marine Research scientists are leading the international testing and application of RNAi technology.
Frank Filippi/CSIRO Livestock Industries

Technology benefits to agriculture

Despite on-going debate about their long-term effects, first-wave genetically modified (GM) crops are now delivering substantial savings and increased profits to farmers, as well as environmental benefits through reduced pesticide use. By allowing lower-tillage systems, herbicide-tolerant GM crops have reduced fossil fuel consumption and erosion, and improved soil structure, fertility and moisture content. Now, RNAi promises to help create a new wave of GM crops that should directly benefit consumer health and nutrition.

Crop geneticists are already using RNAi to develop experimental 'nutraceutical' crops, genetically re-programmed to produce healthier foods. They will help ward off cardiovascular disease, cancer, diabetes, and neurodegenerative disorders like Alzheimer's and Parkinson's diseases.

CSIRO Plant Industry's Dr Allan Green, who developed a new polyunsaturated oilseed crop, Linola, from inedible flax (linseed) in the 1980s, is using RNAi gene-silencing as part of a genetic 'makeover kit' for oilseed crops like canola, cottonseed, sunflower and safflower.

The kit will consist of several RNAi 'silencer genes' to block unwanted enzymes in the crops' oil-synthesis pathways, and various new genes cloned from marine algae. The geneticists will be able to create new oilseed varieties containing oils enriched in the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). Both are essential for normal development and function of the human brain and eyes, and also protect against cardiovascular disease.

Omega-3 fatty acids are currently extracted from marine fish, so with most of the world's major marine fisheries either collapsed or under extreme pressure, these new crops should be a timely, sustainable source of this key nutrient.

RNAi technology could also remove allergenic proteins from foods such as peanuts and wheat, or create completely new food crops by removing toxins from wild crops. Chickling vetch, a dietary staple in some areas of southern Asia and Africa, contains a neurotoxic protein that, with chronic consumption, causes limb paralysis. The crop could be rendered safe with RNAi modification.

Disease firewalls

In 1997 Dr Peter Waterhouse discovered that the presence of viral double-stranded RNA in plant cells induces a virus-quelling effect by the plant. When a virus infects a plant cell, tiny 'nanomachines' called RISC (RNA-induced silencing complexes) are activated. This reaction and targeting information is then exported to neighbouring healthy cells, spreading the protective effect slowly throughout the plant.

The implications were enormous. Assuming this mechanism was common to all plants, 'designer' hairpin genes could protect virtually any plant against any known virus. Sequences from several viruses could even be combined to create RNAi 'firewalls' against the most common virus diseases of particular crops.

Plant virus diseases cause enormous production losses in food crops around the world. In Australia alone, two closely related cereal viruses – Barley Yellow Dwarf Virus and Cereal Yellow Dwarf Virus – can cause yield losses between 15 and 25 per cent in wheat, barley and oat crops in some areas, at an annual cost of tens of millions of dollars.

Plant Industry researchers have constructed an RNAi 'transgene' containing gene sequences common to both viruses, that could protect all cereal crops against both diseases.

Preventing yield losses in cereal crops with anti-viral transgenes would increase food production without requiring more wild land to be cleared, conserving the environment and potentially feeding millions more people. It would also reduce the volume of pesticide used to control aphids and other sap-sucking insects that spread these crop viruses.

Maize and cotton crops protected by insecticidal *Bt* transgenes have already reduced the volume of broad-spectrum insecticides sprayed on these crops by at least 50 per cent in Australia, the US, China, India and South Africa, while substantially increasing yields.

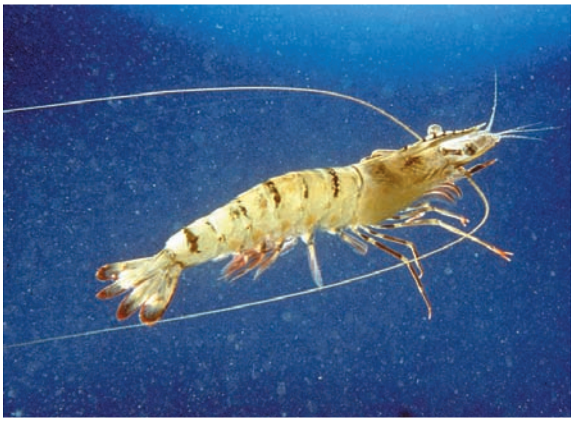
The world's population is predicted to increase by at least 50 per cent by mid-century. Adequately nourishing some nine million people will require a doubling of global food production. Doubling the area under cultivation would consume most of the world's remaining wilderness areas; productivity increases are the only real option.

RNAi for aquaculture

Aquaculture and mariculture are increasing rapidly around the world, providing another source of precious dietary protein for many nations, and reducing pressure on dwindling stocks of wild fish and crustaceans.

Wherever animals are intensively farmed, pathogens and parasites proliferate. It happens in aquaculture too. Waterborne pathogens – viruses, bacteria, fungi and

Right: The giant tiger prawn (*Penaeus monodon*) is the dominant prawn species farmed in Asia and Australia. Scientists at CSIRO Livestock Industries are applying RNAi to combating prawn whitespot syndrome virus (WSSV) which plagues aquaculture.
CSIRO Marine Research.



protozoans – are the bane of prawn farms in Australia and South-East Asia.

Vaccines have not been considered an option for farmed prawns and oysters, because these animals were thought to lack an adaptive immune system. But Dr Marielle van Hulten of CSIRO Livestock Industries in Brisbane told the *Horizons in Livestock Science* conference that new research indicates that crustaceans can mount an antibody-type immune response, and possess other defenses like anti-microbial and anti-viral molecules. They also have RNAi-type anti-viral protection.

Van Hulten's team is exploring the possibility of using RNAi technology to immunise prawns against a potentially devastating pathogen, prawn whitespot syndrome virus (WSSV). Mortality can range as high as 100 per cent, but prawns that survive infection develop immunity.

One option her research team is exploring is to use a harmless prawn virus, IHHNV, ubiquitous in farmed prawns worldwide, as a delivery system for RNAi constructs, which would program the prawn's viral response mechanism to destroy WSSV and other aquaculture viruses.

RNAi for insect pest control

Canadian molecular geneticist Dr Steve Whyard, of the University of Manitoba, and formerly of CSIRO Entomology, told the September conference the 'spreading silence' phenomenon (the RISC-type reaction) also occurs in insects.

He said RNAi technology could yield powerful, precise new ways of controlling insect pests that would avoid the environmental fallout and health hazards associated with broad-spectrum synthetic pesticides.

An RNAi gene-knockdown technique, he said, allowed scientists to rapidly identify potential targets for novel pesticides that will block pests' life-cycle transitions, inhibit feeding, or disrupt fertility and mating behaviour.

Entomologists now have access to extensive libraries of gene sequences, copied from genes expressed in tissues like the gut, nervous system, reproductive system or salivary glands of a number of major insect pests, such as the Queensland fruit fly (*Batrocera tryoni*), sheep blowfly (*Lucilia cuprina*), cotton bollworm (*Helicoverpa armigera*), and notorious carriers of human disease like the malaria mosquito (*Anopheles aegyptoides*) and common housefly (*Musca domestica*).

Nematode worms are prolific parasites of livestock. RNAi approaches have the potential to reduce the downstream effects of current chemical controls on the environment.
CSIRO Entomology

RNAi now allows molecular geneticists to interpret the genomes of pests and benign insects alike, by switching off genes one by one, observing the resulting effects, and, where pests are concerned, looking for vulnerabilities.

At the time of the conference, the CSIRO Entomology project had identified 77 new insect genes as potential targets for pesticides. They are variously involved in digestive physiology, neural function, reproduction and development.

One of the most promising candidates is a gene called *Vasa*, which codes for an enzyme essential for fertility. Although it is active in both sexes, it has sex-specific roles: it mediates egg production in females, and sperm production in males. Reducing *Vasa* expression would sterilise insect pests.

By silencing another gene, for the moulting hormone ecdysone, geneticists can potentially disrupt the transitions between egg, larva, pupa and adult phases of pests' life cycles, effectively stopping reproduction.

Several research agencies around the world, including CSIRO, are exploring the radical possibility of protecting crops with RNAi 'molecular pesticides'.

A crop's cells would be programmed to synthesise RNAi molecules that then target vital genes in the cells of a pest insect's gut. Expressed in the crop's tissues or sap, they would be ingested by leaf-chewing caterpillars or sap-sucking insects, triggering apoptosis – programmed cell 'suicide' – and causing the pest to starve.

RNAi pesticides remain only a theoretical possibility: a major challenge is to engineer them to persist long enough in the hostile environment of the insect's gut to have effect.

Controlling livestock parasites and disease

Veterinary parasitologists are using RNAi-mediated gene silencing to search for new, more environmentally benign ways of controlling livestock pests and parasites.

Dr Ian Sutherland, of CSIRO Livestock Industries in Rockhampton, Queensland, told the conference that the genetic complexity of livestock parasites like nematodes, flatworms and ticks has frustrated efforts to develop safer, environmentally sustainable control measures.

He said most parasites have complex, multi-stage life-cycles, and even if they can be controlled inside the host, they can still change during their free-living stages outside the host.

Because tropical cattle breeds like Brahman and Droughtmaster are more resistant to ticks than temperate breeds like Hereford and Angus, Sutherland's team is conducting RNAi gene-silencing experiments in cell cultures to determine the genetic basis of this difference, hoping to use the knowledge to boost resistance in temperate breeds.

Similarly, Dr Jody Zawadzki's research team at Primary Industries Research Victoria is hoping to develop alternatives to the toxic drenches used to control intestinal worms in sheep, like the barber's pole worm (*Haemonchus contortus*) – a relative of the free-living nematode worm (*C. elegans*).

Efforts to develop anti-worm vaccines have so far failed. Adult sheep develop a degree of natural immunity to *Haemonchus*, but by then have usually shed enough parasites in their manure to infect the next generation of lambs who graze the same paddock.

Sheep dung still contains residues of the powerful drenches used to control intestinal worms and can therefore be lethal to dung beetles, earthworms and other soil fauna, and can leach into surface waters to kill aquatic vertebrates. The drenches also impose strong selection pressure for resistant *Haemonchus* worms. Zawadzki highlighted an urgent need for new, environmentally sustainable control measures.

Her team is using RNAi gene-knockouts to explore the function of anonymous genes, expressed during worm's parasitic and free-living phases, which may offer targets for novel drugs or vaccines to disrupt its life cycle.

The most feared of all livestock diseases, foot and mouth disease (FMD) is extremely contagious. Dr Jef Hammond, a senior researcher with CSIRO Livestock Industries, said that a single infected pig sheds enough virus to infect 100 million other animals. The 2001 FMD outbreak and mass livestock slaughter in Britain cost the UK economy an estimated A$18 billion.

FMD affects about 70 different species of cloven-footed animals, including deer, cattle and sheep. Current vaccines merely suppress the symptoms of FMD, and infected animals continue to shed the highly contagious virus.

In the absence of an effective vaccine, Dr Hammond suggested a ingenious solution: protecting healthy animals with an inhaled spray containing RNAi molecules designed to prevent the virus replicating in the respiratory tract while animals develop natural immunity.

When CSIRO researchers working in Britain experimentally infected mice with FMD virus and treated them with an RNAi molecule designed to suppress viral gene expression, 80 per cent survived. The promising research continues.

RNAi for feral pest control

Three CSIRO divisions conducted the national agency's 'Sterile Ferals' program: Marine Research, Entomology and Sustainable Ecosystems. The program experimented with RNAi gene silencing, using it for genetically mediated control measures of aquatic and marine species grown for aquaculture in Australia.

Marine Research's Dr Peter Grewe said an RNAi-mediated technique called functional sterility could also be used to render aquaculture species sterile outside the aquaculture/mariculture environment, to prevent escapees – especially transgenic species – establishing feral populations or genetically polluting wild stocks.

Tropical-bred Brahman cattle. They have built-in resistance to parasites such as ticks, which scientists hope to exploit through RNAi's capabilities.
CSIRO Livestock Industries

Functional sterility is mediated by an RNAi transgene that would disrupt some gene essential to normal larval development of the fish, crustacean or oyster species in question.

A synthetic compound added to food, but absent from the natural environment, would inactivate the RNAi 'silencer' gene as long as the organism – say, a farmed salmon – remained in the closed environment of the aquaculture farm. Escapees would automatically be rendered sterile.

Dr Ron Thresher's Marine Research team has recently tested the functional sterility technique in the Pacific oyster. Only three per cent of larvae survived escape, compared with 70 per cent in the untreated control group.

The Sterile Ferals project led to a spin-off program, 'Daughterless Carp', currently funded by the Murray-Darling Basin Commission. It aims to develop RNAi-based sterility and sex ratio manipulation to control marine and aquatic pests, like European carp, which is devastating Australian inland waters.

In fish, sex hormones control sexual development. For female larva, an enzyme, aromatase, converts testosterone to estrogen, and the fish develops as a female rather than a male. Without aromatase, the larva 'defaults' to the male development pathway.

In the carp program, mass-reared male carp carrying the anti-aromatase RNAi transgene would be released into carp-infested rivers and waterways to 'seed' wild populations. Wild female carp mating with the transgenic males will then produce only more male offspring – hence the term 'daughterless'. All males arising from these matings would inherit the 'daughterless' gene and transmit it to their own progeny, slowly breeding the carp population to inviability, and a crash.

In RNAi, geneticists have a found a remarkably timely and flexible new tool with which to tackle regional and global scale problems. Its application in the context of sustainable practice approaches is especially promising. Given RNAi works in conjunction with an organism's own defence mechanisms, researchers are pushing forward with confidence and excitement about realising the technology's full potential.

More information:
Gene silencing and RNAi:
www.abc.net.au/catalyst/stories/s799730.htm