Rust busters

Plant geneticists are seeking to combat fungal pathogens by fortifying wheat with 'designer' resistance genes borrowed from other species. **Graeme O'Neill** reports.

Mankind's great leap forward, as far as archaeologists can determine, occurred almost 10 000 years ago when a tribe of hunter-gatherers from Turkey's southern Anatolia region turned from harvesting vast stands of wild einkorn wheat to sowing it.

Blackened grains recovered from ancient settlements in foothills of the Karacadag Mountains are a mixture of wild einkorn, *Triticum monococcum* ssp. *boeoticum*, and its domesticated descendant, *T. monocuccum* ssp *monococcum*, one of the so-called 'land races' of wheat. Small patches of wild einkorn still grow there, on undisturbed ground, between crops of domesticated varieties.

It is unlikely the Anatolian einkorn farmers were troubled by diseases such as wheat stem rust, which plagues modern wheat farmers, because they enjoyed free 'genetic insurance'. Their crops were tiny ponds in a sea of genetic diversity; the same breezes that carried microscopic spores of stem rust fungi brought regular infusions of resistance genes, in pollen grains from wild einkorn.

Today, vast monocultures of highly uniform wheat sprawl across rural Australia's landscapes, genetically and geographically isolated from their ancestors by almost half a hemisphere. These modern wheat cultivars are vulnerable to 'breakthrough' mutants of stem rust fungus.

Molecular geneticist Dr Tony Pryor, of CSIRO Plant Industry at Canberra, puts the problem in stark terms: 'It takes breeders about five years to introduce a new resistance gene into a wheat variety. It usually takes the fungus three to five years to break through.'

Pryor says fungal pathogens of cereals fall into two basic classes: nectotrophs, such as the maize blight fungus *Helminthosporium*, which kill the plant and feast on its dead tissues, and biotrophs such as wheat stem rust, *Puccinia graminis* ssp. *tritici*, which leave the host plant alive and plunder its production.

It's difficult for breeders to stay ahead of biotrophs, because of the way the host plant and the pathogen interact, Pryor says.

Classical genetics has shown the plant recognises some factor made by the rust

Dr Tony Pryor and Dr Nick Collins in the maize nursery at CSIRO Plant Industry. Pryor's research team is working to supplement conventional breeding with transgenics as a source of disease resistance.

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Rust parasites on the leaves of a flax plant.

fungus, probably a secreted protein, made by one of the toolkit of genes employed by the fungus to enter the host cell. The as-yet unidentified genes that make these factors are called avirulence genes.

Plant species 'learn' to recognise the product of the avirulence gene, and to activate their defensive mechansims. When, through chance mutation, the avirulence gene is modified or deleted, the fungus again has the ascendancy. The plant must 'relearn', again through chance mutation, to recognise the fungus and mount a defence.

Over aeons, plants and their pathogens have become locked into an ultimately unwinnable arms race. Each breakthrough mutant challenges the plant to respond with a gene capable of recognising a new or modified avirulence factor; each time the plant counterpunches, the fungus must create a new mutant.

But given that rust fungi produce prodigious quantities of spores, the numbers are stacked overwhelmingly on the pathogen's side. It's usually only a matter of time – three to five years – before one spore among billions comes up with a novel mutation that founds a 'breakthrough' strain, forcing the breeder to look for a new resistance gene.

Yet plants, which inherently have a far smaller potential to produce new, resistant mutants, persist in a hostile world of fast-mutating pathogens. Pryor says this implies that resistance genes in plants have some special property that allows change in order to meet the new challenge. Since a plant cannot anticipate how a pathogen might change, it cannot 'design' new genes. In any case, evolution is a design-free zone: natural selection simply sieves the products of mutation or genetic recombination until it stumbles across something that works.

The emergence of new rust resistance genes in wheat and its relatives, however, is clearly not an annual event, nor even a three to five yearly one. So, in the late 20th century, an ominous problem looms for wheat breeders: the pool of available resistance genes is fast drying up.

Since late last century, when William Farrer bred the first rust-avoiding wheat cultivars on his farm, 'Lambrigg', on the banks of the Murrumbidgee near Canberra, (Farrer cleverly bred cultivars that matured before the main rust season), breeders have been restricted to species sexually compatible with wheat. These include the ancient land races of wheat, including einkorn and emmer wheats, and all their close cousins such as barley and rye. Lately, breeders have been casting their nets into ever-more remote gene pools, including a few wild grasses that can also be coaxed into improbable liaisons with wheat. These include *Agropyron* and various species of *Aegilops*.

But wheat breeding now stands on the cusp of its greatest revolution, one that will allow breeders to harvest an ocean of genetic diversity. They will be able to vault the interbreeding barriers that separate wheat and other cereals from their thousands of relatives in the megadiverse plant family, the Gramineaceae, or perhaps even to exploit resistance genes from virtually any plant species in the world.

And where nature cannot provide, molecular geneticists will be able to construct 'designer' resistance genes, cutting and splicing nature's own designs to create new defensive weapons unknown in nature.

Pryor says he and his colleagues, and their long-time collaborators in the United States – Dr Scott Hulbert's team at Kansas State University – are close to realising a prediction made in 1978 by Plant Industry chief, Dr Jim Peacock, and a colleague, Dr Bill Scowcroft.

In 1978, Peacock and Scowcroft upset a generation of plant breeders by predicting that transgenic plant breeding would eventually make their craft obsolete by providing an unlimited source of resistance genes. Their prediction came only five years after the first successful gene-splicing experiment in bacteria in 1973, and five years before the creation of the first transgenic plant of any kind.

Recruiting jumping genes

Pryor and his colleagues began their search for rust-resistance genes in maize (*Zea mays*) in 1980, but like plant molecular geneticists around the world, found themselves chasing green unicorns in the sparsely mapped forests of crop plant genomes.

Nobody knew how resistance genes worked, so they didn't know what to look for, but they did have some idea of where to look.

Hunting resistance genes

Technique 1: Transposon tagging

NEARLY 50 years ago, US maize geneticist and Nobel laureate Dr Barbara McClintock proved the existence of mobile genetic elements in the maize genome that could modify supposedly fixed traits in plants, in apparent defiance of the Mendelian rules of inheritance.

McClintock identified a binary system of transposable elements, or transposons, consisting of a 'master' element called Ac (activator) and smaller 'slave' elements called Ds (dissociator).

It is now known that the master element Ac encodes a DNA-cutting enzyme called a transposase, which excises Ac itself and its Ds slave elements from their current sites in maize chromosomes. The mobile elements 'jump' to new locations – often into the DNA sequences of nearby genes –- disrupting their activity.

Ac can be transferred, by conventional hybridisation, into plants with dormant Ds elements scattered throughout their genetic blueprints. Ac 'wakes' the Ds slaves, and they 'jump' to new sites.

By screening large numbers of Ds mutant plants, geneticists can identify those that might contain genes of interest. For example, if a formerly rust-resistant line has spontaneously lost rust resistance, it can be assumed that a Ds element has gone into the gene that confers resistance to that rust race.

Only one copy of Ac master transposons usually goes into the plant. Its unique DNA sequence flags the location of the disrupted gene, and provides a 'handle' for a gene probe to retrieve the transposon. When Ac is retrieved, some of the flanking DNA sequences from the disrupted gene come out with it. These can be used as gene probes, to progressively recover contiguous sequences from the gene, until its full DNA sequence has been recovered.

Ds, unfortunately, does not always provide a useful marker or handle. Often, there are simply too many copies present in the plant genome to determine which particular Ds element has disrupted a gene of interest. The CSIRO team encountered this problem after producing their rust-susceptible Ds mutants of maize.

Technique 2: Polymerase chain reaction (PCR)

Once several genes belonging to a new family of genes with a related function, such as disease-resistance genes, have been cloned and sequenced, molecular geneticists can use the polymerase chain reaction (PCR) to search for other genes of similar function.

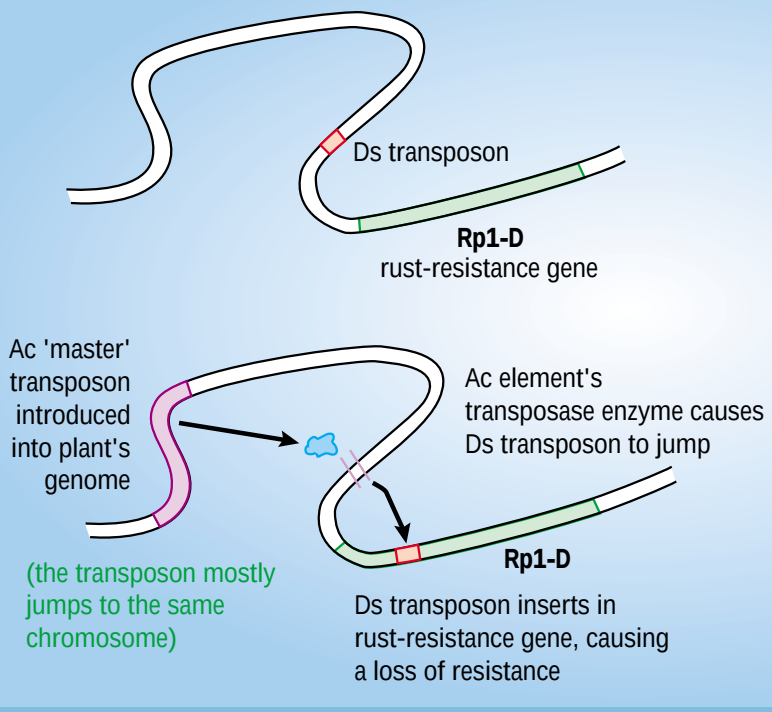
Genes of related function typically share highly-conserved DNA sequences: 'hand-me-downs' from an ancestral gene.

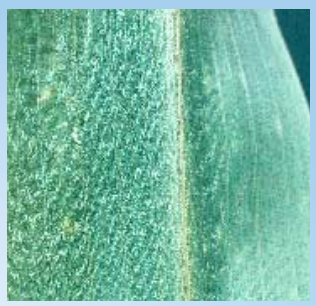
From these highly-conserved sequences, such as the nucleotide-binding site (NBS) of the flax-rust resistance gene, researchers can design special DNA 'tags', called primers, to search for similar sequences using the polymerase chain reaction.

The primers seek out and attach to related sequences on chromosomes. Two primers are effectively bookends for the DNA sequence between them. Researchers then use an enzyme called DNA polymerase to 'photocopy' the intervening DNA. The copied DNA sequence may be similar or identical to the reference sequence. Alternatively, it may be from a related gene that shares the primer sequences.

The PCR technique complements other gene-search techniques. It can only be used if researchers have the appropriate primers, derived from the original gene.

Transposon tagging





A leaf from a rust-resistant plant.

A leaf from a Ds-disrupted mutant; resistance is lost.

Jeff Ellis and Val Ryle in the flax tissue culture room. Flax is used by the Plant Industry research team as a model for gene-transfer experiments.

Model plants

WHEAT is Australia's major cereal crop, and Australia is one of the world's biggest wheat exporters. So why choose maize and flax, rather than wheat, as experimental subjects?

For much of this century, maize has been intensively studied by classical geneticists. Detailed data on chromosomal locations of many important traits provided a 'mud map' to guide molecular geneticists in a much finer-grained exploration of plant DNA. Wheat, by comparison, was *terra incognita*.

But even if Dr Tony Pryor and his colleagues had found a rust-resistance gene in maize in the 1980s, they could not have transferred it into non-resistant maize. All cereals, including the world's most important crops – maize, rice and wheat – proved reluctant patients for the gene surgeon's scalpel until Japanese geneticists finally succeeded with rice in 1993.

The hitch is that the plant genetic engineer's courier, the crown-gall bacterium *Agrobacterium tumifaciens*, only infects dicots, such as flax.

In the act of infecting its host, *Agrobacterium* inserts a small 'cassette' of genes, called a Ti-plasmid, into the cell nucleus, reprograming the cell to nourish the microbe. By splicing new genes into a disarmed Ti-plasmid that does not cause the disfiguring symptoms of crown gall disease, plant gene surgeons can introduce new traits and regenerate whole plants from the transformed cells.

Genetic engineers have since managed to persuade *Agrobacterium* to transform rice, barley and maize, but not wheat, Australia's major crop. Plant Industry scientists have been able to transform wheat cells with a 'gene gun' that fires golden bullets – microparticles of gold coated with DNA – through the tough cellulose cell wall of wheat cells, but the technique is unreliable.

Flax was their choice as a model for gene-transfer experiments because of pioneering work by US geneticist Harold Flor, of the University of North Dakota, on the genetics of flax-rust resistance.

Pryor says the choice was also influenced by the presence in Australia of an endemic species of flax, *L. marginale*. The species is widespread in Australia, and populations from different regions are a rich source of new resistance genes for transfer into cultivated flax, *L. usitatissimum*, thus extending the model as a test of wild, non-commercial relatives as a source of resistance genes for agronomic crop plants.

US maize geneticist Virginia Rhoades had shown in 1935 that the genetic locus Rp1, which gives resistance to maize rust, *Puccinia sorghi*, was located near the tip of the short arm of chromosome 10. Some 30 years later it became clear that the locus was genetically complex: several rust-resistance genes probably existed there.

In the early 1980s, knowing nothing about the function of resistance genes, and, not wanting to make assumptions, Pryor and his colleagues decided they would use transposon tagging to see if they could knock out a resistance gene in a model plant, flax (see story on page 29).

Transposons are mobile genetic elements that move around the DNA of their host by self-excising from one location on their chromosome and re-inserting at another. The antics of these 'jumping genes' are known to be the cause of spectacular plant mutations such as variegated flowers and leaves.

Half a century ago, Nobel laureate plant geneticist Dr Barbara McClintock identified two such jumping genes in maize. The genes, activator (Ac) and dissociator (Ds), work in tandem: Ds cannot 'jump' without the help of Ac. (See Hermes and the housefly, *Ecos* 91, pp22-25.) In flax plants Ds occurs naturally, but Ac does not.

In an effort to pinpoint the rust-resistant gene in flax, Dr Jeff Ellis, Dr Jean Finnegan and Dr Greg Lawrence transferred Ac from maize into a flax plant known to have a particular kind of rust resistance: L6. They then produced a quarter of a million plants in the hope that, in at least one of them, Ac and Ds might jump into the resistance gene, upsetting its function.

Upon screening the plants for resistance to the flax rust, *Melampsora lini*, they found a plant that had lost its resistance, a sign that Ac and Ds had performed as hoped. In 1993, the embedded Ac element was used as a retrievable marker to clone the resistance gene. (It was designated L6 decades earlier by US plant pathologist Harold Flor.) This was the first rust-resistance gene from a plant to be cloned anywhere in the world.

The first plant genes for virus and bacterial diseases were cloned in 1994, a watershed in the hunt for plant disease-resistance genes. That first trickle has now become a steady stream. Dozens of resistance genes for viruses, bacteria and fungi now have been cloned from a diverse range of crop plants, including the plant geneticist's 'green mouse', the tiny mustard relative *Arabidopsis thaliana*.

One of Pryor's colleagues, Dr Evans Lagudah, has isolated a gene called Cre-3 (resistance to cereal root eelworm) from goat grass, *Triticum tauschii*, which, when transferred to wheat, confers resistance to the cereal cyst nematode *Hetrodera avenae*.

A common defence

Given the diversity of plants and their pathogens, marked differences exist in the DNA sequences of resistance genes. Yet many of the proteins encoded by these genes feature a recurrent theme, with variations, in their amino

acid sequences: a motif called NBS-LRR, possibly the legacy of an ancient pathogen-defence system.

NBS-LRR stands for nucleotide-binding site, leucine-rich repeat. The LRR domain is variable, but is punctuated by sequences carrying multiple repeats of the amino acid leucine. In contrast, the NBS domain tends to be conserved between different types of resistance genes, and across plant species, suggesting it serves some general-purpose role in the plant's defensive repertoire.

Pryor and his colleagues suspect the LRR domain serves as a receptor – a molecular detector – for the avirulence protein of a pathogen, while the NBR domain is a docking site for the host plant's internal signalling proteins.

A plausible sequence of events begins with the pathogen invading the host cell and secreting a protein – the avirulence factor – into its cytoplasm. The resistance gene's receptor protein, drifting in the cytoplasm, detects the avirulence protein and wraps its LRR domain around it, somehow altering the nucleotide binding sequence. The NBS domain can now bind (activate) signalling proteins. The activated proteins transmit an alarm signal down into the cell's nucleus, activating a suite of genes that mobilise the plant's defences.

The rust fungi that attack the world's major cereal crops all belong to the genus *Puccinia*. When attacked, resistant wheat, maize and barley varieties all defend themselves with the same 'scorched-earth' strategy: the plant activates gene products that kill off the cells in the immediate vicinity of the infection. Since the fungus can only survive in living cells, the hypersensitive response effectively quarantines the invader.

This hypersensitive response leaves the plant's leaves flecked with patches of dead tissue. Highly-resistant plants develop only tiny flecks, while fully-susceptible plants show little necrosis and little restriction of pathogen growth.

The L6 flax rust resistance gene (mentioned earlier) induces a necrotic response in flax leaves, similar to rusts in maize and wheat. In experiments to determine how a flax rust resistance gene 'recognises' a specific race of flax rust, Ellis and Lawrence grafted onto the NBS region of L6 the LRR receptor domain from another flax-rust resistance gene called L2, which is specific for a different race of flax rust.

A strong necrotic response was induced in the 'designer' L6/L2 resistance gene that protected flax plants previously lacking rust resistance against L2-type rust attack. In this way the experiment dramatically confirmed that the specificity of the resistance gene can be encoded in the LRR domain.

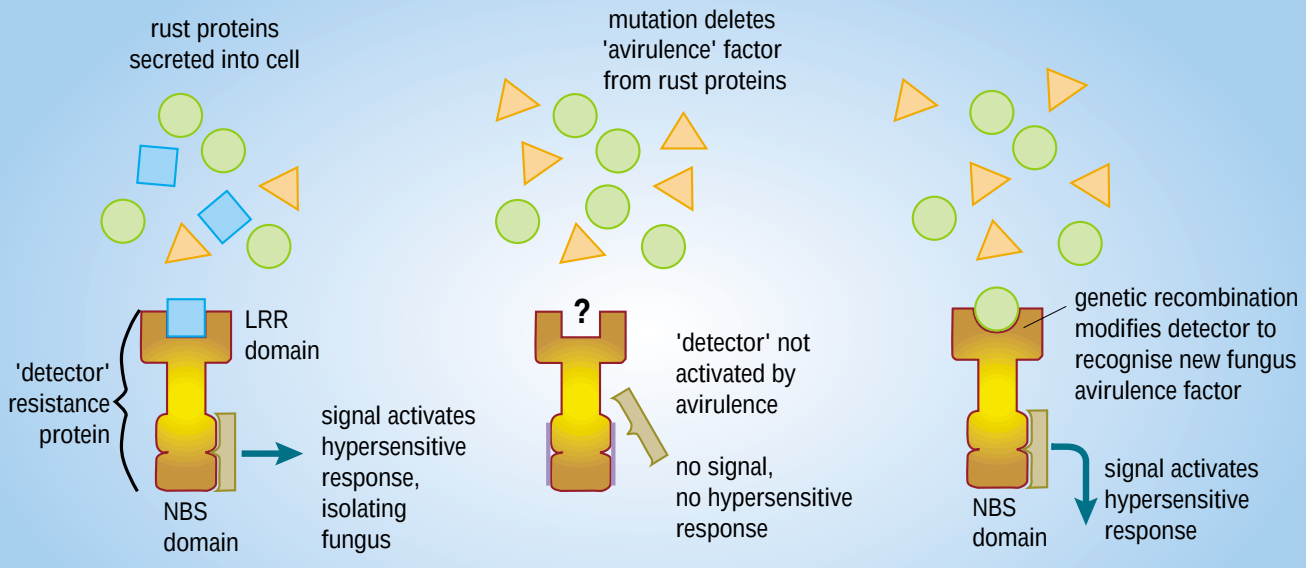
The implication of this discovery is that elite cultivars that have succumbed to a breakthrough rust strain could be rapidly resuscitated by grafting a new LRR domain, selected (or designed) to recognise the new strain, onto the failed resistance gene.

Zeroing in on maize

It was the discovery that many disease-resistance genes carry NBS-LRR domains that allowed Pryor's team to clone the first rust-resistance gene from a cereal: the Rp1-D rust-resistance gene from maize.

They knew the gene resided in the Rp-1 locus on chromosome 10, and among the 200 000-odd maize plants that they had transposon-tagged in the 1980s were several that had lost Rp1-D-type resistance, presumably because the Rp1-D gene had been disrupted by a Ds transposon. But a

Plant/pathogen interactions



Rust proteins are secreted into the plant cell to allow the rust to grow. When a rust protein is recognised by plant 'detector' resistance protein, it becomes known as the 'avirulence' factor.

host of Ds transposons now peppered the chromosomes of the mutant plants. There was no way to selectively retrieve the one embedded in the Rp1-D gene.

A member of the team, Dr Nick Collins, resorted to a Polymerase Chain Reaction (PCR) search of the maize plant using DNA primers derived from the NBS domain of known resistance genes, such as the L6 flax-rust resistance gene (see story on page 29). The primers were to detect virtually any gene containing the NBS motif. They were in luck. The PCR sweep detected a match – or rather, a number of matches – near the tip of the short arm of chromosome 10.

Pryor's team used a DNA-cutting enzyme to dissect the tip of chromosome 10 in a rust-resistant maize variety, and obtained about eight distinct fragments of DNA. The PCR product confirmed that each one contained an NBS motif, so the fragments presumably contained eight closely related genes. But which one was the Rp1-D rust-resistance gene?

They then performed the same dissection on a Ds-mutant maize that had lost Rp-1 resistance. Again, they obtained eight fragments. This time, however, one of these fragments was swollen by 400 base pairs of DNA: precisely the size of a Ds transposon. In a 'revertant' plant that that had reacquired Rp1-D-type rust resistance, the fragment had returned to its original size – Ds had fled, restoring the disrupted gene to normal function.

In the same week, Hulbert's team at Kansas provided independent confirmation that the gene was indeed the Rp1-D rust-resistance gene. They had gone down the same route, but had used a different transposon called Mu (Mutator) to generate their own maize mutants.

Pryor's team cloned and sequenced the Rp1-D gene, and confirmed that it had the typical NBS-LRR motif: they had cloned the first cereal rust-resistance gene.

Using the maize Rp1-D gene as a probe, the Plant Industry researchers have now identified a corresponding gene in barley, *Hordeum vulgare*. The gene also appears to be part of a cluster of about half a dozen genes, analogous to those at the Rp1-D locus in maize.

The fact that, in both species, similar genes occur in clusters or small gene families of related sequence hints at a mechanism for creating new resistance genes for emergent rust mutants.

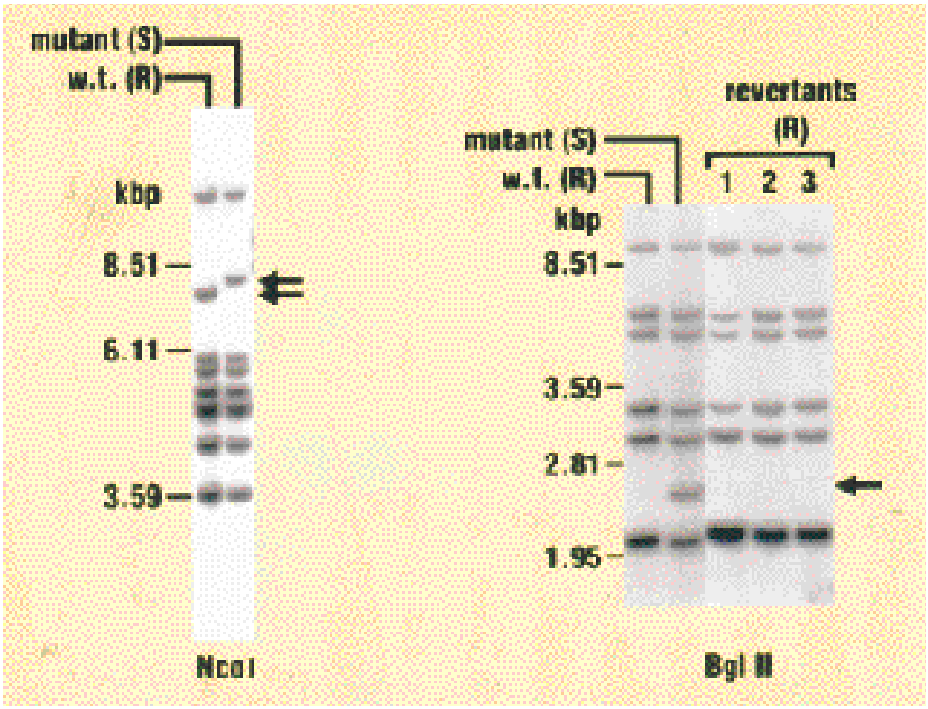
When genes with closely-related DNA sequences lie close together on a chromosome, they can swap segments by a mechanism called unequal recombination. Rarely, a new, recombinant resistance gene, by chance, will confer protection against rust strains to which the parent plants lacked resistance.

Australia's multi-billion dollar wheat industry cannot afford to wait for natural recombination; human-assisted recombination is the way ahead.

'We now have eight or nine resistance genes, including a second resistance gene from flax, the M gene,' Pryor says. We're in a position to ask a number of questions. Do maize rust resistance genes work in barley and wheat, and vice versa? Or, a more outlandish question: will a monocot resistance gene work in a dicot, and vice-versa?'

Molecular geneticists may also be able to construct synthetic resistance genes for cereals, from components designed by nature, such as the FIS (flax inducible sequence) isolated by Pryor's team five years ago (see story on page 33). These might include fungicide genes that would be activated by pathogens themselves in the act of attacking plants.

'One of the critical observations made by Jeff and Greg Lawrence was that a susceptible cultivar of flax could be made resistant with a transgene for the flax L6



Eight fragments of maize DNA from the tip of chromosome 10 dissected with a DNA-cutting enzyme. The first lane shows DNA from a wild-type resistant plant. Lane 2 shows DNA from the Ds mutant maize where one of the eight fragments has been increased in length by 400 base pairs (bp) due to the insertion of the Ds1 transposon.

abstract

The speed with which biotrophs such as wheat stem rust, *Puccinia graminis* ssp. *tritici*, mutate is outpacing conventional plant breeding. Molecular geneticists at CSIRO Plant Industry, using transposon tagging, were the first to locate and clone a rust-resistance gene from flax. The discovery of a common motif, NBS-LRR, contained in DNA sequences of resistance genes of different species raised the possibility that elite cultivars of non-resistant wheat could be fortified with a resistant version of the motif, either cloned from another species, or manufactured synthetically. The discovery led to the location and cloning of a rust-resistance gene from maize, the first from a cereal species. A corresponding gene in barley has since been identified.

Keywords: wheat; wheat stem rust; stem rust fungi; fungal pathogens; plant breeding; genetic engineering; resistance genes.

Greg Lawrence with flax plants. Lawrence and his colleagues produced a quarter of a million flax plants and used transposon tagging in the hope of identifying a rust-resistance gene. The experiment has led to the cloning of the first rust-resistance gene from a plant.

rust resistance and the resistance shown was the precise specificity of the original L6 gene,' Pryor says. 'Until then, nobody knew that rust-resistance transgenes would work.'

'It was proof of the concept that all of us have been dining out on since Peacock and Scowcroft made their prediction in 1978 that transgenics would one day supplement conventional breeding as a source of disease resistance.

'History has shown the average durability of a rust resistance gene is three to five years. Breeders are walking a tightrope.

'But with transplanted resistance genes, we should be able to restore resistance in a wheat cultivar in little more than six months, without disrupting its elite genes, having added novel resistance genes never previously available.'

More about fungal resistance

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A fuse-lighter called FIS

FIVE YEARS ago, Dr Tony Pryor's team initiated a project to isolate a flax gene that was activated, or induced, by rust infection.

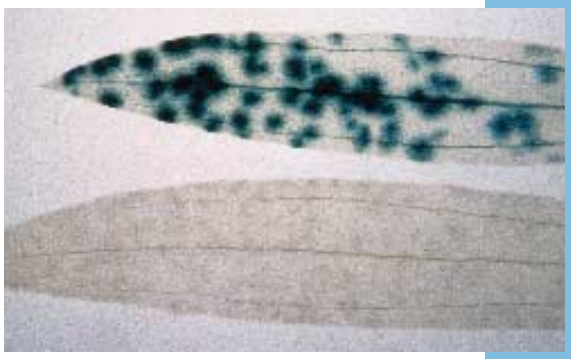
They found such a gene, and cloned its promoter – the DNA 'switch' that is directly activated by rust attack. The promoter, which they called FIS (flax inducible sequence) has been patented jointly by CSIRO and the Australian National University.

In an experiment that demonstrated the potential of designer resistance genes, the CSIRO researchers linked the FIS promoter to a standard 'reporter' gene called GUS. This encodes an enzyme called fl-glucuronidase, causing tissues in which the reporter gene is expressed to stain bright blue.

They inserted the GUS gene, under the control of with the FIS promoter, into flax, and then challenged the resulting transgenic plants with flax rust. When they stained leaf sections, blue halos appeared around the infection sites. The FIS promoter had been directly activated by the rust infection, and switched on the GUS gene.

Pryor says the FIS promoter could be grafted onto other genes, such as those for natural plant fungicides, allowing plants to respond directly to fungus attack by synthesising a lethal dose of fungicide in their leaves. This active response would complement the plant's own, passive, necrotic response.

Even with their phenomenal propensity to mutate, rust fungi would find it difficult to break through such 'stacked' defensive mechanisms.



Two susceptible rust-infected flax leaves, both stained for GUS activity. The upper leaf is from a transgenic plant containing the GUS reporter gene under the control of the rust-inducible FIS promoter and shows blue halos of reporter gene activity surrounding each site of infection. The lower leaf is from a non-transgenic control plant.