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Making plants make more food

An understanding of plant nutrient transport and reproduction systems will enable the creation of higher yielding and more nutritious grains.

With global food demand destined to rise by 50% in the next 15-20 years, crops are needed that grow more efficiently on limited arable land. **Graeme O'Neill** chronicles the quest to control mechanisms of plant reproduction and growth.

Somewhere in the chromosomes of *Arabidopsis thaliana* lies a gene – or a few genes – that allows plants to set seed without fertilisation. The apomixis gene has evolved as a survival trait of last resort in higher plants that reproduce by seed; when pollination fails, apomictic plants simply create seeds that become clones of their parents.

In September 1997, Dr Abed Chaudhury's team of researchers at CSIRO Plant Industry announced they had produced mutant *Arabidopsis* plants that take them close to isolating the apomixis gene. Chaudhury's team is part of the division's Gene Regulation and Plant Development group, led by Dr Liz Dennis.

Apomixis could be worth $7-$8.6 billion to world agricultural production, according to conservative estimates by the Australian Centre for International Agricultural Research (ACIAR), a sponsor of the CSIRO project.

If today's farmers planted the seeds of their hybrid crops they would end up with an unsaleable mess. Every seedling would carry its own combination of the best and worst traits of its grandparents, some of which may have blown in from wild populations growing nearby. This phenomenon, called segregation, destroys the crop's quality and uniformity.

Seeds from apomictic plants, however, yield successive generations with the same characteristics as the original hybrid parent. Given this capability, the world's main cereal crops – rice, wheat and maize – would be easier to improve and maintain. Not surprisingly, plant breeders have dreamed of developing apomictic crops since the dawn of systematic plant breeding last century.

CSIRO's apomixis project was born four years ago when Chaudhury and Plant Industry chief, Dr Jim Peacock, outlined a novel strategy to identify and clone the gene in *Arabidopsis,* a crucifer commonly used as a genetic 'model'. It was based on the likelihood that apomixis was a regulatory gene that switches on early in the sequence of genetic events that normally ensues when a pollen grain fertilises an ovum.

The idea was to identify a gene that operates early in the seed-development pathway after pollination, and trace its connections to identify the master gene that 'decides' to switch to the 'last-resort' apomixis pathway after pollination fails.

This required finding an entry point into the apomixis pathway, preferably just above the point where the apomixis gene switches on. Chaudhury and his colleague Dr Ming Luo started out with a male-sterile mutant *Arabidopsis.* The flowers of this plant lack male organs, so it cannot self-pollinate. Chaudhury and Luo induced the mutant to produce seeds using pollen from a normal plant.

They then exposed the resulting first generation (F1) seeds to a chemical mutagen, ethyl methyl sulfate, and grew the seeds on to produce an F2 generation. Among the plants grown from the F2 seeds was one that goes part-way to producing seed without being fertilised. It produces seed consisting of endosperm tissue, and a tiny embryo that aborts before completing its development. The mutant is thus a partial apomict: it starts to produce seed apomictically, yet unlike natural apomictic plants, it produces no viable seed.

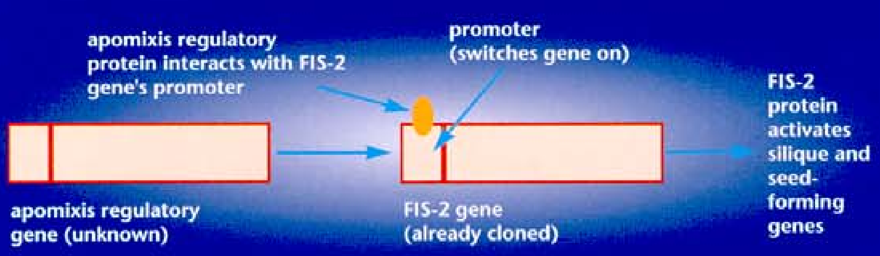
Any plant that produces seed – whether by pollination or by apomixis – needs somewhere to store and protect its seeds while they develop. In *Arabidopsis,* a tiny, pod-like structure called a silique fulfils this role.

In the original male-sterile mutant, the siliques begin to develop, but arrest if it is not fertilised by pollen from another, normal plant. The unfilled siliques remain short: an easily-visible character for selection. But if the plant is cross-pollinated, the siliques elongate to accommodate the seeds.

Chaudhury was looking for a male-sterile plant that developed long siliques without being pollinated. This would indicate that the plant had produced seed by apomixis, rather than via a pathway induced by pollination.

During the screening process, Chaudhury's team found another mutation where the silique is long, but devoid of seed. This parthenocarpic mutant has spun off another opportunity for CSIRO breeders. Dr Anna Koltunow, of the division's Adelaide laboratory, has been trying to isolate the gene responsible for parthenocarpy, which would be useful for producing seedless fruit, such as man-

Dr Abed Chaudhury and Dr Ming Luo of CSIRO Plant Industry have isolated a gene that controls the initial steps seed development.



Cloning the apomixis regulatory gene

1. Link the FIS2 gene promoter to the reporter gene so it will switch on when the apomixis gene is active.

2. Insert this gene into the *Arabidopsis* apomictic mutant. The reporter gene will cause the plant tissues to stain blue when it is activated by the apomixis gene protein.

3. Mutate this apomictic plant, aiming to inactivate apomixis gene – the tissues will no longer stain blue because the reporter gene is no longer switched on.

3. Screen these plants for mutant genes – one will be the apomixis gene.

4. Use DNA sequences from the mutant gene to locate and clone candidate apomixis genes.

5. Test proteins made by candidate gene(s) for the ability to activate the FIS2 gene.

Learning from botany's 'green mouse'

ARABIDOPSIS, a tiny crucifer related to cabbage, cauliflower and rapeseed, is an ideal genetic model for crop plants. It germinates, grows, flowers and sets seed in just eight weeks, and it has a compact genome. It also has fewer genes than most crop plants, making it easier to search for genes. In the past decade, it has become the subject of intense scrutiny by plant geneticists.

In laboratories around the world, molecular geneticists are dissecting the tiny plant's genome, cataloguing the estimated 25 000 genes on its five chromosomes, and constructing a master map of their location and arrangement. Each week, new genes from *Arabidopsis* further illuminate the genetic mechanisms of plant development and growth, and speed the search for valuable new genes in crop plants.

darins and table grapes, which generally command a premium on international markets.

Using standard methods of gene isolation, Chaudhury's team isolated a gene that controls the initial steps of silique formation and seed development. They called it FIS2 (Fertilisation-independent Seed 2). They also found two other genes, FIS1 and FIS3, which seem to perform the same function as FIS2.

FIS2 is not the apomixis regulatory gene; it's a regulatory gene that probably is regulated itself by apomixis.

Regulatory genes make special proteins that coordinate the activity of other genes – or whole genetic pathways – by switching them on or off. Genes under the control of the same regulatory protein can usually be identified by some shared DNA motif in their promoters, the region of DNA code upstream from the protein-coding sequence, which switches the gene on (or off) and determines when, where and how much protein it will make.

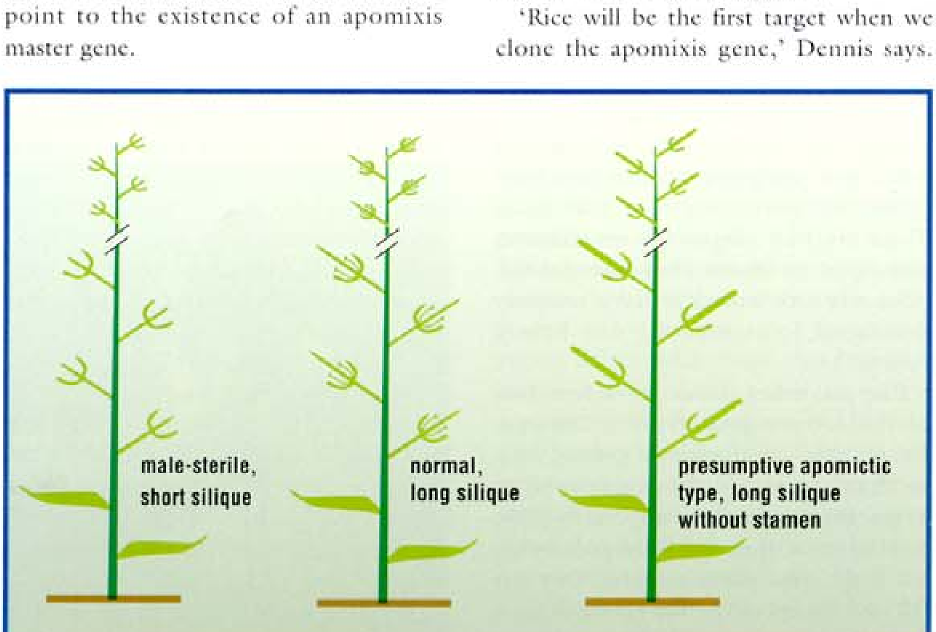
The team is now testing what happens when you combine the different FIS genes – does seed development proceed further without fertilisation?

Dennis says there is good reason to believe apomixis is controlled by a single, 'master gene'. In certain grass species, the apomixis trait traces to a single, specific chromosome segment. Genetic studies of the apomictic daisy, *Heiraceum*, also point to the existence of an apomixis master gene.

Topping up the rice bowl

Growth in world food production, particularly of staple grains, is showing ominous signs of falling behind population growth. Rice production must double within the next 20 years. With most arable land already accounted for, the only way to meet the demand is to increase yields. Driven by this need, the apomixis gene project evolved as a partnership between CSIRO, ACIAR and the International Rice Research Institute (IRRI) based in the Philippines.

'Rice will be the first target when we clone the apomixis gene,' Dennis says.



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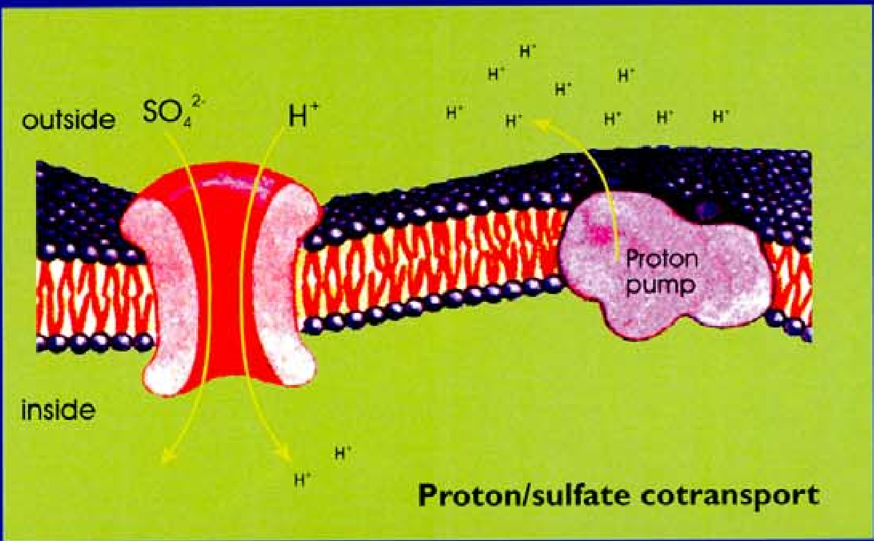

Proton power

SULFATE, nitrate and phosphate transporter genes are strikingly similar in structure and, presumably, in function.

The proteins always contain 12 hydrophobic domains, each having approximately 23 amino acids. The particular amino acid 'recipe' differs between genes. With their affinity for the lipid membrane, these domains anchor the protein as it loops through the lipid membrane.

Viewed from the cell's exterior, the loops form tight helixes, and the helixes group to form a doughnut-shaped structure with a pore at its centre. The pore traverses the membrane, and nutrient ions, packaged with protons to neutralise their negative charge, are drawn through the pore into the cell's interior.

A proton pump in the membrane delivers protons onto the outer surface of the membrane, maintaining a voltage difference between the outer and inner surfaces of the membrane.

'It's an excellent model for other cereals, because there's already a reliable system for transforming rice with new genes, and an international project is under way to produce a physical map and complete DNA sequence for the rice genome.'

Rice is an ideal candidate crop. Unlike wheat and maize – which have been extensively hybridised during the past century – most of the world's rice is still grown by traditional, subsistence techniques, using varieties that have been grown for centuries, even millennia. These are well adapted to the climates and soils in which they are grown. (Chinese rice breeders have recently developed some superior new hybrid varieties.)

Rice and other cereals are all members of the diverse grass family, Poaceae. Dennis says several pasture grasses, such as *Pennisetum* and the maize relative *Tripsicum*, are facultative apomicts. Like most grasses, they are wind pollinated, but if the wind doesn't deliver, they can fall back on apomixis. The apomixis gene from *Arabidopsis* should allow molecular geneticists to isolate apomixis genes from these grasses, which also could be used to transform rice and other cereals.

Asia's traditional rice *Oryza sativa*, is an outbreeder; it does not self-pollinate, requiring pollen from adjacent plants to set seed. Asian farmers traditionally reserve some of their harvest for the next season's crop. If new hybrid rice varieties – or even different varieties from other regions – are grown near traditional varieties, their pollen could fertilise the traditional varieties, disrupting their genetic integrity.

But if the traditional varieties were transformed with the apomixis gene, they would be immune to the effects of outbreeding. Meanwhile, apomictic hybrids grown near traditional varieties would keep their genes to themselves, allowing rice farmers to evaluate new, higher-yielding or more disease-resistant varieties in different environments, without compromising their venerable neighbours.

Dennis says apomictic fixed hybrids would probably be superior to traditional varieties, but it would be possible to give traditional varieties the advantages of fixed hybridity, so they would come true from seed.

Apomixis would provide similar benefits for wheat and maize farmers who could grow the same variety year after year, confident that it would always come true from seed. This capability would profoundly change the direction and economics of the hybrid seed industry, because farmers would no longer be dependent on hybrid seed producers. But, as Dr Dennis points out, there will always be a demand for new hybrid varieties with characteristics such as improved yield and disease resistance, or superior taste or baking properties.

The apomixis project is a long-term enterprise, but Chaudhury believes that once the gene has been isolated, it could take as little as five years to introduce it into the first crop plants, depending on the state of genetic transformation systems for those crops.

Wheat is potentially problematic. As Australia's most important cereal crop, it would be the preferred target after rice, although producing wheat with new genes is still difficult technically. But Dennis is confident that by the time the apomixis gene becomes available, transforming wheat will be relatively routine.

The benefits of apomixis would not end with cereals. Chaudhury says apomixis could be introduced into fruit and vegetables, or to any other crop where fixed hybridity is required.

Pumping ions

Another piece of plant 'magic' that has taken scientists many decades to decipher is the uptake and transport of essential nutrients such as phosphate, nitrate, sulfate and potassium. Just how do plants spirit these negatively charged ions through the lipid membrane that encapsulates all living cells?

It's a basic law of physics that like charges repel. Being negatively charged on its inner surface, the lipid bilayer of the cell membrane is naturally impermeable to ions, but particularly to anions, which must be actively pumped through the membrane into the cell's interior.

In classic studies four decades ago, Professor Emmanuel Epstein of the University of California showed that plants have two systems for taking up nutrients: a high-affinity system that operates when nutrient levels are low, and a low-affinity system that comes into play when nutrients are at high levels.

Later research found the systems were based on the combined handiwork of protons and proteins embedded in the cell membrane. The protons carry a positive charge that neutralises the net negative charge of anions, enabling 'transporter' proteins to draw them into the cell (see diagram at left).

But in the age before recombinant DNA technology, Epstein could only infer the existence of the membrane-bound molecular pumps that supply plants with nutrients. Details of their identity, structure and mechanisms remained a mystery.

In 1993, a strategy for locating and cloning membrane transporter proteins was devised by Dr Frank Smith's research group at CSIRO Tropical Agriculture and British scientists at the Long Ashton Research Station at Bristol. The strategy involved knocking out the gene in yeast which enables it to take up sulfate nutrients, then inserting genes from plant roots into this yeast to see which one would restore its power to take up sulfates.

In February 1995, after screening more than 600 000 yeast samples, Smith's team cloned the first root-specific, high-affinity anion transporter genes from the tropical legume, *Stylosanthes hamata*. Two of the genes, SHST1 and SHST2, encoded sulfate transporters. A third gene isolated by the team, SHST3, encoded a low-affinity sulfate ion transporter.

By April 1996, Smith's group had located and cloned two phosphate transporter genes from *Arabidopsis*, (APT1 and APT2) and by October 1997 had cloned a sulfate transporter gene, HVST1 from barley. Other scientists in Australia and the UK are working to pinpoint nitrate and potassium transporter genes.

Short-circuiting the system

By studying transporter proteins and their control systems, molecular geneticists may be able to bypass the feedback mechanisms in plants limiting nutrient uptake.

For a plant to grow, develop and complete its life cycle, it must maintain its internal nutrient concentrations within an adequate, safe range. Over hundreds of millions of years, the cells of terrestrial plants have evolved internal feedback loops to maintain a stable supply of nutrients. These operate under soil conditions in which the absolute concentration of nutrients can vary widely, and pH levels can limit their availability to the plant.

Smith says the wild ancestors of modern crop species evolved mechanisms allowing them to use nutrients conservatively. Some of these conservative traits have been lost in the process of selection and breeding. For example, there is evidence that modern crops use fertilisers less efficiently than their ancestors.

A crop such as sugarcane typically requires annual applications of 120-240 kilograms of nitrogen, 30-50 kg of phosphorus, 40-100 kg of potassium and 15-25 kg of sulfur. The aim of manipulating nutrient-uptake mechanisms would be to produce crop plants that use expensive fertilisers more efficiently, thereby reducing costs to farmers.

Studies at Smith's Brisbane laboratory have shown that when roots of the tropical legume Siratro (*Neonotonia atropurpureum*) are deprived of an external sulfate supply for 24 hours, their potential sulfate uptake rate increases six-fold, rising to nine-fold after 48 hours. But within eight hours of the sulfate supply being restored, their sulfate uptake potential declines to that of normally nourished plants. Responses to phosphate deprivation and resupply are similar, but much slower: it takes the plant two or three days to return to normal after being resupplied with phosphate.

Dr Frank Smith's team has cloned genes responsible for transporting sulfate and phosphate around plants.

More nutritious fruit and leaf vegetables could be developed by manipulating the genes controlling ion transport.

A key question is whether this relatively rapid 'tuning' of nutrient uptake to match nutrient supply is due to changes in the activity of the sulfate transporter protein. Or does the gene's output change, to reduce or increase the number of sulfate pumps in the cell membrane?

Smith says it is now clear that the major tuning takes place at the level of the gene itself. Its production of transporter proteins varies over periods of minutes to hours. But plant cells also seem to possess a fine-tuning mechanism that responds almost instantly; feedback mechanisms modify the shape of the transporter proteins, slowing or accelerating the flux of anions into the cell.

It is likely that proteins from other genes interact with the transporter gene's promoter – the region containing its DNA 'switches' – to increase or repress its activity. Smith says the transporter genes are probably members of a complex system of feedback loops that fine-tune nutrient levels in the cell as sulfate availability ebbs and flows in the root zone, either as a result of changing soil moisture, or after fertiliser has been applied. All these systems have a dual transport mechanism, just as Epstein described 40 years ago.

Smith says genetic surgery on the promoters of the nutrient transporter genes would render them insensitive to their internal control mechanisms, bypassing the feedback loops that normally limit nutrient uptake. If the high level of potential uptake observed in nutrient-deprived plants could be maintained in the presence of adequate nutrient levels, it might be possible for plants to accumulate nutrient stores early in their life cycle, and make use of them during flowering and seeding.

In practice, this involves introducing another copy of the transporter gene, linked to a promoter insensitive to the feedback mechanisms limiting nutrient uptake. Smith and his colleagues are experimenting with modified sulfate and phosphate transporter genes.

Smith says another strategy would be to make more efficient use of the nutrients already taken up by the plant, by rapidly remobilising nutrients held in tissues that are no longer contributing to plant growth. Remobilised nutrients must pass through other membranes in the xylem and phloem to reach cells in actively growing tissues, and nutrient transporter proteins must mediate this process.

'If we can clarify how plants move nutrients around themselves, it will open the way to create more nutritious grains, fruit or leaf vegetables,' Smith says. 'We might, for example, raise calcium content in grain to help prevent osteoporosis, the iron content of vegetables to reduce the risk of anaemia, or the sulfur content of grain to improve the quality of bread.

'We may be able to develop special plants or algae with high nutrient uptake as biological filters for sewage effluent. And we may be able to prevent food crops from taking up undesirable substances, such as heavy metals or salt.'

Is there a risk that tinkering with the green machine's inner workings might have the opposite effect to that desired? Smith acknowledges that geneticists will have to be careful to avoid perturbing the natural control mechanisms; there must be a sound understanding of the biological effects of manipulating the nutrient transport system. But his group's own experiments have already shown that a new promoter that de-represses the sulfate transporter gene dramatically increases sulfate uptake, and increases the level of sulfate stored in the plant's tissues, with no apparent ill effect.

'A lot of evidence is now emerging that nature operates sub-optimally,' he says. 'At the October meeting of the Australian Society for Biochemistry and Molecular Biology, several papers described how induced mutations can make these genes more active than the wild-type genes.

'As long as we understand the biological effects of these changes, and make the changes carefully, we can literally speed up evolution in important food and fibre plants by improving on what nature has already achieved.'

Smith is now working with Germany's Max Plank Institute for Molecular Plant Physiology to clarify what happens at the atomic scale when plants absorb and transport nutrient ions.

abstract

Plant geneticists at CSIRO have produced mutant *Aradidopsis* plants that take them closer to cloning the apomixis gene, the regulator of fixed hybridity. Control of this mechanism would make food crops easier to improve and maintain. In other research, sulfate and phosphate transporter genes have been cloned. This may lead to the short-circuiting of internal signals limiting nutrient uptake, enabling development of plants that are higher yielding, more nutritious and designed for specific uses.

Keywords: apomixis regulatory gene; *Arabidopsis*; cereal crops; nutrient transport systems; plant breeding; proton pump; transporter genes.

More about ion transport

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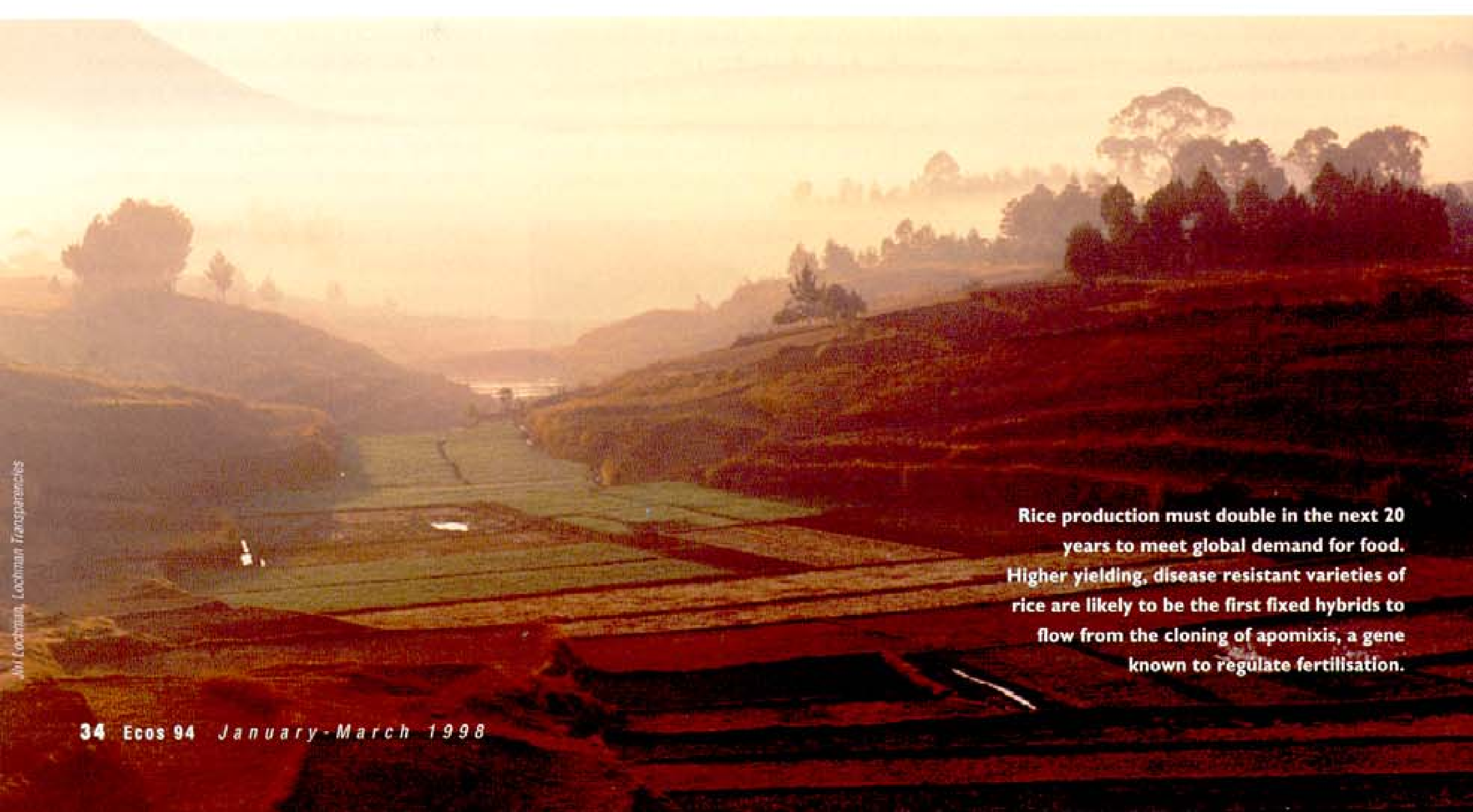

Rice production must double in the next 20 years to meet global demand for food. Higher yielding, disease resistant varieties of rice are likely to be the first fixed hybrids to flow from the cloning of apomixis, a gene known to regulate fertilisation.

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