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# Terminator Technologies

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## Terminator Technology In New Guises

*The terminator technology, which genetic engineers harvested seeds not to germinate, was vigorously opposed by farmers and consumers all over the world. One of the claimed benefits is that it prevents the spread of transgenes, but its real purpose is to protect corporate patents on seed. It offers no benefit to farmers or consumers. In response to widespread opposition, Monsanto has recently announced that it will not commercialize terminator seeds, if only because such seeds are not yet available. But research is continuing.*

In fact, terminator technology has continued to be developed under a number of different guises. The main one is 'Genetic Use Restriction Technologies' (GURT) ( see Traitor Tech. The Terminator's Wider Implications. *RAFI Communique*, Janurary/February, 1999). Newer versions make seeds dependent on the application of a chemical for germination, or for expressing the desired transgenic trait – the chemical being exclusively manufactured by the company selling the seeds, so the end result is again to protect corporate patents.

Genetic engineering is not a precise technology. On the contrary, it is uncontrollable, unreliable and unpredictable (see Viral Gene Switch – A Recipe for Disaster?). The GURT technologies are worse. They depend on 'site-specific recombination' – breaking and joining DNA at two specific sites recognised by a recombinase enzyme, which then snips out the DNA between the two sites. The two sites might flank a blocking sequence within a promoter, so removal of the blocking sequence will enable a gene to be expressed which makes a poison to prevent the seed from germinating, for example. Or it may do the opposite, the sites may flank the promoter itself which is necessary for expressing the gene, so when it is removed, the gene will no longer be expressed, and the seed will germinate. The gene coding for the recombinase enzyme is engineered to be under the control of the external stimulus, ie, the chemical manufactured exclusively by the company, so the recombinase will be active only when the chemical is applied.

Thus, GURT technologies involve multiple feats of precise gene stacking, inserting the gene stacks into plants in exactly the configurations constructed, and subsequent to that, precise regulation in the transgenic plant(s), and exactly predictable response of the recombinase to the external chemical stimulus. However, those requirements for precision are beyond the capability of the genetic engineer. The hazards of the transgenic DNA resulting from GURT technologies are much greater, because the imprecisions of inserting multiple gene-constructs are multiplied, and also because of the site-specific recombination mechanisms deliberately introduced. Recombination creates new combinations of genes and has the potential to scramble genes and genomes when it is imprecise. It is already known that recognition between designated recombination sites and their enzymes (recombinases) are far from exact (see Kohli et al, 1999, *The Plant Journal* 17, 591) and many mistakes are anticipated. These genes – the recombinase and the recombination sites - once engineered into the plants, will spread both by ordinary cross-pollination and by horizontal gene transfer, multiplying the opportunities for scrambling or rearranging genes and genomes.

Now, a new report claims to have used site-specific recombination to solve one problem of imprecision in genetic engineering plants, which is that multiple copies of the foreign genes tend to be inserted at each site (Srivastava, V., Anderson, O.D. and Ow, D.W. (1999). Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proc. Nat. Acad. Sci, USA* 96, 11117-21). As pointed out by the authors, multiple copies are undesirable because it often leads to transgene instability, either on account of gene-silencing by the host organism, or structural instability such as recombination between multiple copies which result in the loss of the transgene.

To solve the problem, the researchers constructed vectors consisting of transgenes flanked by recombination sites that are *inverted* (so they are not recognized by the recombinase). When the transgene is integrated into the wheat genome in a repeated configuration, the recombinase will only snip out the block of genes between sites that are in the same orientation, so ultimately, only one single copy of the transgene will be left at each site, at least in theory. However, it is clear that many unexpected results were also obtained alongside the expected, including the inversion of genes (literally genes turned around), scrambled transgene configuration and scrambled host genome, often arising from the imprecise action of the site-specific recombinase. The authors admit the possibility of scrambling and removing host genome DNA in their procedure, and recommends obtaining single-copy transgenic lines from several different progenitor lines.

In our view, genes and constructs involving site-specific recombination systems should not be approved for release into the environment in any form**.**

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**Terminator Gene Product Alert**

So far, attention has focussed on the terminator technology without considering the extremely toxic gene product, barnase, which could be a major health hazard.

Barnase is the gene product in terminator technology which prevents harvested seeds from germinating. It is a ribonuclease (RNAse), an enzyme that breaks down RNA indiscriminately, isolated from the soil bacterium, *Bacillus amyloliquefaciens*. The enzyme is normally lethal in the living cell, but is produced in the bacterium together with an inhibitor. This inhibitor is separated from the enzyme when it is excreted. Traces of barnase are toxic to the rat kidney (1) and to human cell lines (2).

In some terminator constructs, Barnase is linked to a plant promoter active only in the cells of the tapetum ( the sac from which pollen cells are generated). The pollen cannot develop when the barnase gene is active ( Us patent 5,723,765 is jointly held by the United States Department of Agriculture(USDA) and Delta and Pine Company, which for a time considered joining Monsanto). The terminator technology was designed to control seed production to benefit seed companies by preventing seed saving. The system has also been studied for use in seedless fruit production (3).

The barnase component of terminator is also being used to produce male sterile lines in wheat by introducing promoters from corn or rice linked to barnase gene (4). Male sterile lines are used to produce hybrids which are more uniform than inbred lines and may also show heterosis (hybrid vigor). Hybrids also benefit seed producers because saved seeds segregate undesirable progeny. Several methods have been studied for producing hybrid canola including the use of barnase.

The most significant question about use of barnase is: do the crops bearing barnase gene pose any threat to humans or animals? This question does not seem to have been addressed by those developing or testing the crops. During seed production, barnase may be present in dust and debris from the crop and surfaces, along with groundwater may be contaminated with the toxin. Humans or animals breathing the plant material may experience severe toxicity. Normally, for crop generation both the barnase gene and the gene for a barnase inhibitor are required. It seems likely that mitotic recombination could easily separate barnase gene from barnase inhibitor gene. Such complications should not be ignored.

Assuming that commercial hybrids are created , say for example, in canola, the hybrid crops are likely to produce viable pollen. The pollen would likely segregate active tapetal barnase producing some male sterility in weedy relatives and neighboring canola producers fields. In a sense the hybrid producers would pollute the crops of neighbors in a severe manner, much like the pollution of conventional canola from GM pollen in Saskatchewan. Except that the barnase gene produces a well known toxin active against humans and animals.

1. Ilinskaya,O and Vamvakas,S (1997). Nephrotic effect of bacterial ribonucleases in the isolated and perfused rat kidney. *Toxicology* 120, 55-63

2. Prior,T, Kunwar,S and Pastan,I (1996). Studies on the activity of barnase toxins in vitro and in vivo. *Biocong Chem*7,23-9

3. Varoquaux,F, Blanvillain,R, Delseny,M and Gallois,P (2000). Less is better: new approaches for seedless fruit production. *TIBTECH* 18,233-43

4. DeBlock,M,Debrouwer,D and Moens,T. (1997). The development of a nuclear male sterility system in wheat. *Theor and Appl Gen* 95,125-31

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ISIS News 7/8, February 2001, ISSN: 1474-1547 (print), ISSN: 1474-1814 (online)

**Terminator Crops are here!**

**ISIS Exclusive**

*When Monsanto announced they would give up terminator crops more than a year ago, everyone heaved a sign of relief. Meanwhile, the UK and US governments are colluding with industry to bring them back, and what's more, they have already been field tested in Europe, UK and Canada beginning in the 1990s.*

Terminator technology is so named by its critics (thanks to RAFI) because it genetic engineers seeds to be sterile, for no other purpose than to protect and enforce corporate patents on GM seeds.

ISIS discovered that Aventis' spring and winter GM oilseed rape (canola) currently field tested in the UK are actually engineered with the 'terminator technology'. Moreover, similar crops

have been field tested in Europe, Canada and the US since the early 1990s, and several have been commercially released.

The technology was promoted simultaneously on both sides of the Atlantic towards the latter half of 2000. The USDA solicited public comment on the technology itself with the recommendation that it could be used to prevent gene flow. The UK government, meanwhile, carried out a public consultation exercise on a draft document, "Guidance on Best Practice on the Design of GM Crops" produced by the Advisory Committee on Releases to the Environment. That document presents the technology as one of the main methods for preventing gene flow, and thereby improving the safety of GM crops

The technology is ineffective in preventing gene flow, and it makes use of two very dangerous genes that should never, never be released. One is a universal cell poison, and the other scrambles genomes. Read the full story in "Killing Fields near You", this issue.

## Killing Fields near You
## Terminator Crops at Large

*'Terminator technology' makes harvested seeds sterile in order to enforce corporate patents on GM seeds. The public first became aware of the technology in patents jointly owned by US Department of Agriculture and Delta and Pine Land Company, but no one suspected that terminator crops actually exist. ISIS discovered, however, that terminator crops have been field-tested in Europe since 1990, and in the United States since 1992, where several have already been released commercially. The US and UK Governments are now promoting terminator crops as a means of preventing GM genes from spreading.* ***Mae-Wan Ho****,* ***Joe Cummins*** *and* ***Jeremy Bartlett*** *explain why the technology is not only ineffective in preventing gene flow, but introduces extra, potentially fatal hazards. They must be stopped immediately.*

Last December, while acting as an expert witness in defence of citizens who have taken action against GM field trials in the UK, MWH discovered that among the crops are AgrEvo's 'male sterile' GM oilseed rape. At the time, we were preparing our submission to a public consultation document, "Guidance on Best Practice in the Design of GM Crops" sent to us by the UK Government's Advisory Committee for Release to the Environment (ACRE). One of the main 'enabling technologies' for 'best practice' – to prevent gene flow - is to engineer seed or pollen sterility.

It soon dawned on us that the GM oilseed rape undergoing field trials have been created using 'terminator technology', so named by critics because it can render harvested seeds sterile in order to enforce corporate patents on GM seeds. AgrEvo's application revealed that such crops have been field-tested in France and Belgium since 1990, and subsequently, in Sweden and Canada, before coming to the UK. We further discovered that similar male sterile lines have been tested in the US at least as early as 1992 and several already released commercially.

The complete terminator system requires two components, the enzyme barnase, which kills the germ cells, and a 'site-specific' recombinase, which is used to block the action of barnase until it is required. There have been 132 field trials of crops with barnase [1], the vast majority without risk assessment. Crops modified for male sterility include rapeseed, corn, tobacco, cotton. Brassica oleracea, potato, poplar, chicory, petunia and lettuce. The USDA commercial release data include 4 crops with barnase: a corn and a canola by AgrEvo, a chicory by Bejo, and another corn by Plant Genetic Systems [2]. Separately, the 'site-specific' recombinase has been engineered into corn and papaya, and there have been 14 field trials between 1994 and 1998, without environmental impact assessment, as it was deemed unnecessary.

There are more than 150 US patents listing barnase or site-specific recombinase or both [3] the oldest going back to 1987. The first terminator patents to catch public attention were those jointly owned by US Department of Agriculture and Delta and Pine Land Company, which Monsanto had intended to acquire. The novelty in those patents is the proposal to combine the barnase with the site-specific recombinase, giving the company complete control over the hybrids as well as proprietary chemicals that control gene expression.

These patents met with universal condemnation world wide, as being contrary to basic human rights. The technology would prevent farmers from saving, replanting and exchanging seeds, practices going back thousands of years that are essential to food security. Monsanto announced it would give up terminator crops, to everyone's relief. Research and development, however, have continued unabated.

At the same time that consultation on best practice is occurring in the UK, the US government is consulting its citizens on the USDA-Delta and Pine patents, which the USDA plans to develop commercially. The USDA is also recommending the technology for preventing gene

flow. At the end of January 2001, the USDA signed a deal with Delta and Pine for commercial development to go ahead [4].

Surely, to require containment of GM genes is to admit that they are unsafe, which is a reason for stopping GM crop development altogether, and not for promoting a morally bankrupt technology. Moreover, this technology introduces serious potentially fatal hazards [5] in the genes used. Barnase is a universal poison, while the recombinase can scramble genomes in unpredictable, lethal ways.

**Denial or just being kept in the dark?**

When confronted by a journalist, a spokesperson from the UK Department of the Environment, Transport and the Regions (DETR) denied that the enzyme barnase is in the crops undergoing field trials. He was reported to have said that the barnase gene, and not the enzyme, was present in "a few oil seed rape crops", and that "where the enzyme would be poisonous, the gene was not harmful."[6] Obviously, he forgot that the barnase gene had to be expressed to make the barnase enzyme in order to cause male sterility.

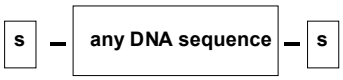
Dr. Ian Woiwod of Rothampstead, a scientist involved in overseeing the UK field trials, indicated that he had no knowledge of such crops in the field trials [7]. Indeed, a correspondence describing the trials published in *Nature* [8] made no mention of the male sterile spring and winter oilseed rape. During a workshop at the first meeting of the Intergovernmental Committee on the Cartegena Protocol on Biosafety held in Montpellier last December, a UK Government delegate from the DETR actually thanked MWH for providing the information.

**How seed/pollen sterility is engineered into crops**

There are two key components to terminator technology. The first component is literally the 'terminator' barnase, an enzyme that breaks down RNA. RNA is an intermediate in the expression of all genes, and that is why barnase is lethal to all cells in which it is expressed. Barnase is specifically inhibited by barstar. Both barnase and barstar are produced by a soil bacterium, *Bacillus amyloliquefaciens*. Inside the bacterial cell, barstar binds to barnase in a one-to-one complex, disarming the latter so it can do no harm. However, when barnase is secreted outside, it is no longer bound to barstar and is thus harmful to other cells.

The second component is a 'site-specific' recombinase enzyme that recognises specific 'sites', or short DNA sequences, labelled 's' in the diagram below. Any stretch of DNA sequence flanked by two such sites will be spliced out by the recombinase.

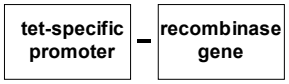
To engineer pollen sterility, the *barnase* gene is placed under the control of a promoter, a gene

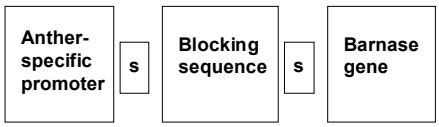



switch that allows the gene to be expressed only during anther development, ie, in the male part of the flower. The barnase with its anther-specific promoter is stitched next to the transgene of interest, say, a gene coding for herbicide tolerance, also with its own promoter. Theoretically, there will be no fertile pollen from this transgenic crop. In the case of crops that are normally self-fertilised, there will be no seeds set. In out-crossing plants, the only fertile seeds set will be those fertilised by non-GM varieties nearby, which will not be herbicide tolerant; so farmers who want the herbicide tolerant trait will have to buy fresh seeds from the company every season.

The problem is that such a male-sterile line by itself cannot be propagated, it does not breed true. To propagate the line, the 'site-specific' recombinase can be used. For example, the promoter of the barnase could be blocked by a sequence flanked by sites recognised by the recombinase.

The recombinase can be engineered into the same GM line with the barnase gene for male sterility. The recombinase is placed under the control of a promoter that responds to an external chemical, say, the antibiotic tetracycline.



When tetracycline is applied, the recombinase is expressed, and splices out the blocking sequence in the barnase promoter, so barnase is expressed. By treating harvested seed with tetracycline before they are sold to the farmer, the company can ensure that the plants grown from the seeds will be pollen sterile.

If female-sterility is required, the barnase gene could be placed under the control of a promoter that works only during ovule development, ie, in the female part of the flower, and the rest is similar.

Alternatively, the recombinase may be engineered into a GM line with the gene coding for barstar, which, when crossed with the male sterile GM line containing barnase, will produce a hybrid. The hybrid, treated with tetracycline, will produce plants that will still set seed, at least in theory, because the barstar inactivates the barnase. However, if the harvested seeds were re-sowed, the farmer will find that only about half (7/16) [9] of the seeds will have the same characteristics as those originally purchased from the company, and about one fifth (3/16) of the seeds may be completely sterile. It could be considerably worse.

Terminator crops cannot prevent gene flow and introduce extra, potentially fatal hazards

Terminator crops are ineffective for preventing gene flow because

a. All gene control systems are 'leaky', in the sense of not being 100% accurate; as a result, some fertile pollen/seeds are likely to be produced.
b. Pollen-sterile GM plants can still be fertilised by non-GM pollen, just as GM pollen from ovule-sterile plants can cross with non-GM plants, thus enabling gene escape.
c. Terminator crops will not limit horizontal gene transfer, if anything, the complicated constructs involved will enhance it. This is a process whereby the GM DNA is taken up directly into cells of unrelated species and incorporated into the cell's genome.

The genes involved in terminator crops are potentially fatal. Barnase is a potent RNAse that breaks down RNA indiscriminately, and is known to be harmful, if not lethal, to all cells, animals and humans included. When perfused into rat kidneys, barnase causes kidney damage [10]. The 'site-specific' recombinases are known not to be specific. There is already evidence suggesting that genomes can be scrambled by the recombinase (see Note 5). This has now been clearly demonstrated.

The recombinase, Cre, catalyses recombination between two *lox* sites, splicing out the stretch of DNA in between. When Cre is expressed at high levels in the sperm cells of transgenic mice, the males became 100% sterile, despite the absence of any *lox* sites [11]. Sterility is caused directly by the recombinase enzyme scrambling the genome, essentially by breaking and rejoining DNA at inappropriate sites on the same or different chromosomes. Embryos fertilised by these sperms arrest predominantly at the 2-cell stage, and do not go beyond the 4-cell stage (see "Terminator Recombinase Does Scramble Genomes", p. ).

The greatest danger of terminator crops stems from the spread of the genes and constructs, not only to related species by out-crossing but by horizontal transfer to unrelated species. The complication of the GM constructs involved will only increase structural instability, which in turn increases the tendency for the genes to transfer horizontally and recombine. Transfer of both the terminator gene barnase and the recombinase will have drastic, potentially fatal effects on agriculture, biodiversity and health.

These killing crops must be stopped once and for all.

1. www.nbiap.vt.edu/cfdocs/fieldtests3.cfm
2. Thanks to Jill Davies for this information rivercare@blackfoot.net
3. www.delphion.com
4. "USDA - Delta & Pine Land Commercialization Agreement Reached, Pollen Transformation System" January 29, 2001 SCOTT, Miss., Jan.26 /PRNewswire/ via NewsEdge Corporation.
5. See "Why patents on life-forms and living processes should be rejected from TRIPS – Scientific briefing on TRIPS Article 27.3(b)" by Mae-Wan Ho And Terje Traavik, TWN and ISIS Report, 1999 www.i-sis.org; "Terminator in different guises" ISIS News #3, December 1999 www.i-sis.org; "Terminator gene product alert" ISIS News#6, September 2000 www4.i-sis.org
6. "GM 'poison' allegation denied" http://uk.news.yahoo.com/001205/79/ar32n.html.
7. Personal communication by e-mail to ISIS from Dr. Ian Woiwod, 8.12.2000.
8. Firbank, L.G. *et al*, (1999). *Nature* 399, 727-8.

9. It deviates from the usual 9/16 Mendelian ratio for the inheritance of two genes, barnase and barstar. On account of a one-to-one complex between barnase and barstar; plants in which there are two copies of barnase to one copy of barstar will be expected to be partially sterile.
10. Ilinskaya, O and Vamvakas, S (1997). Nephrotic effect of bacterial ribonucleases in the isolated and perfused rat kidney. *Toxicology* 120, 55-63.
11. Schmidt E.E., Taylor, D.S., Prigge, J.R., Barnett, S. and Capecchi, M.R. (2000). Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *PNAS* 97, 13702-13707.

## Terminator Recombinase Does Scramble Genomes

*ISIS has predicted that the recombinase enzyme used in terminator technologies will scrambled genomes (see "Terminator in new guises" ISIS News #3, December 1999 <www.i-sis.org>). This has now been demonstrated in transgenic mice engineered with the recombinase.*

The recombinase Cre is part of the 'site-specific recombination' Cre/*lox* system originally isolated from the bacteriophage (bacterial virus) P1. Cre catalyses recombination between two *lox* sites, splicing out any stretch of DNA in between. The *lox* site is a 34 basepair element consisting of 13 basepair inverted repeat separated by a core of 8 basepairs. In order to work, the 8 basepair core of the two *lox* sites have to be in the same orientation.

The system is not only used in plants, but extensively exploited in transgenic mice. Studies in the test-tube have shown that Cre recombinase can catalyze recom-bination between DNA sequences found naturally in yeast and mammalian genomes. These 'illegitimate sites' often bear little sequence similarity to the *lox* element. However, there have been no reports on such illegitimate recognition in the animals or plants themselves. And there have even been pilot studies using the Cre/*lox* system in human gene therapy.

In a new study [1] researchers in the United States showed that high levels of Cre expression in the sperm cells of heterozygous transgenic mice leads to 100% sterility in the males, despite the absence of any *lox* sites. Heterozygous mice carry only one copy of the Cre recombinase gene.

The sterility is caused directly by the recombinase enzyme scrambling the genome, essentially by breaking and rejoining DNA at inappropriate sites on the same or different chromosomes. The researchers have pinpointed the genome scrambling event to the time at which the two 'daughter' spermatids (precursors of sperms) and their paired chromosomes have just separated from each other; but are still joined by a 'cytoplasmic bridge'. This is enough to allow the enzyme to pass from the spermatid containing the recombinase gene to the other which does not, thereby to scramble up the chromosomes of both the transgenic and nontransgenic spermatid. The result is 100% sterility. Embryos fertilized by these sperms arrest predominantly at the 2 cell stage, and do not go beyond the four cell stage.

The researchers warn: "These results indicate that Cre can catalyze illegitimate recombination having overt pathological consequences in animals." A similar recombination system is found in animals containing the RAG recombinases. There, illegitimate recombinations in somatic cells are linked to human leukemias.

1.Schmidt, E.E., Taylor, D.S., Prigge, J.R., Barnett, S. and Capecchi, M.R. (2000). Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. PNAS 97, 13702-13707.

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## Stop Release of GM Insects!

*The United States Department of Agriculture (USDA) received an application to release GM pink bollworm engineered with jumping genes this summer. This is now delayed as a result of legal challenge from civil society.* ***Dr. Mae-Wan Ho*** *and* ***Prof. Joe Cummins*** *expose evidence of instability in these GM insects, and warn of rampant horizontal gene transfer and recombination. Releasing such GM insects is tantamount to giving wings to the most aggressive genome invaders. They argue that the project should not be allowed to go ahead.*

The release was planned to begin July 15 2001 and end July 14 2002 in a small field in Arizona. The pink bollworms are engineered with a jumping gene carrying a green flourescent protein (GFP) from a jelly-fish. The purpose of the release experiment is to use the GFP marker to evaluate the efficacy of sterile insects that are being developed in the hope of eradicating the bollworm pest. The release is being challenged by the International Center for Technology Assessment and Center for Food Safety in Washington DC (www.centerforfoodsafety.org), The application can be viewed at:

http://www.aphis.usda.gov/biotech/arthropod/permits/0102901r/0102901r.html,

and the public are invited to comment by July 23. So please do so! Our detailed submission is posted on ISIS Website, "Comments on: Environment Assessment: Confined field study of a transgenic pink bollworm, *Pectinophora gossypiella*" <www.i-sis.org>.

Geneticists have created a particularly hazardous class of gene transfer vectors for engineering insects. These are transposons, or mobile genetic elements, which, as the name implies, are genetic units that can move from one site to another in the same genome or move between genomes belonging to unrelated species. Transposons are related to viruses and proviral sequences that, like transposons, are found in the genomes of all species.

A transposon consists of several genes flanked by terminal repeat sequences. One of the genes will code for the enzyme transposase, which is necessary for moving the element. However, elements that have lost the transposase gene can nevertheless get help from the enzyme encoded in other transposons. Transposons come in groups, or superfamilies, many of which have members distributed widely across species belonging to different phyla of both animals and plants. These 'promiscuous' trans-posons have found special favour with genetic engineers, whose goal is to create 'universal' systems for transferring genes into any and every species on earth. Almost none of the geneticists has considered the hazards involved.

A group in Boston created a vector from *mariner*, a superfamily of transposons found across genomes of diverse species from insects to plants and vertebrates, including human beings. One element belonging to this superfamily, *Hirmar1*, isolated from the horn fly, was used to make 'mini-transposons' consisting of the short inverted terminal repeats, between which any gene expression cassette(s) can be inserted. The researchers constructed a minitransposon with a kanamycin antibiotic resistance marker gene driven by a bacterial promoter. This minitransposon was found to jump easily into the *E. coli* and *Mycobacterium* chromosome. It is known to recognize the dinucleotide TA. The probability of this dinucleotide occurring in any stretch of DNA is $0.25^2$ or 6.25%. Within the 500 base pairs of the bacterial chromosome analysed, 21 of the 23 possible TA dinucleotide insertion sites were occupied [1].

The experiment shows that the transposon can be stripped down to the bare minimum of the flanking repeats, and it can still jump into genomes. The reason, as mentioned earlier, is that the transposase function can be supplied by a 'helper' transposon. Such helper trans-posons are ubiquitous. So, it would seem obvious that integrated transposon vectors may easily jump out again, to another site in the same genome, or to the genome of unrelated species. There are already signs of that in the transposon, *piggyBac*, used in the GM bollworms to be released by the USDA this summer.

The *piggyBac* transposon was discovered in cell cultures of the moth *Trichoplusia*, the cabbage looper, where it caused high rates of mutations in the baculovirus infecting the cells by jumping into its genes [2]. The *piggyBac* is 2.5kb long with 13 bp inverted terminal repeats. It has specificity for sites with the base sequence TTAA. (The probability of this sequence occurring is $0.25^4$ or 0.4%.) This transposon was later found to be active in a wide range of species, including the fruitfly *Drosophila*, the mosquito transmitting yellow fever, *Aedes aegypti*, the medfly, *Ceratitis capitata*, and the original host, the cabbage looper [3]. The *piggyBac* vector gave high frequencies of transpositions, 37 times higher than *mariner* and nearly four times higher than *Hirmar*.

In another experiment, the *piggyBac* vector, with its transposase gene disabled and carrying the green fluorescent protein gene cassette, was used to transform the silkworm, *Bombyx mori L* [4]. Transposase function was provided by a helper-plasmid containing a *piggyBac* transposon, also disabled, by having one of its terminal repeats removed. The integrative vector and helper plasmids were both injected into silkworm embryos. The adult fertile moths (G0) resulting from the injected embryos were mated in single pairs among themselves or backcrossed to the unmodified parent, and the resultant broods (G1) were analysed.

A total of 2498 embryos from two strains of silk worms were injected, 1164 (46.6%) of the embryos hatched resulting in 654 (26.7%) fertile adults, single-pair matings among which 12 broods (0.5%) expressing green fluorescent protein were found.

The genomic DNA of the broods were analysed with Southern blot (a technique that gives information on the inserts). Here is how the authors reported their results.

"Southern blot analyses of the DNA of transformed G1 insects showed that one to three different inserts were present in a single animal and that larvae from the same progeny [ie, brood] had different insertions. These insertions were inherited independently at the G2 generation…

"The presence of multiple independent inserts in many G1 larvae indicates that a single gamete from the G0 parent can harbor several insertions and that different gametes can have different insertions. Eighteen insertions were observed in 12 G1 individuals issued from three

transformed parents. It is likely that this result underestimates the total number of insertion events that occurred in the G0 moths." (p.82)

Why were there such a large number of different inserts? There were two possible explanations.

"Either the integration events [of the *piggyBac* vector] in the germ line occurred late during development [of the injected embryo]", so that the same adult carries a population of germ cells each with different insertions, "or successive rounds of transposition took place after an initial insertion event". The latter hypothesis, considered more likely, "would explain why – despite the low frequency of insertion in the parental population [0.5%] – the number of inserts is high in the transformed insects….. A similar situation was also observed in transgenic *C. capitata*, and it was also attributed to secondary mobilizations of an initial single insert." (p.82)

In other words, there is evidence that the inserts had moved between the G0 and the G1 generations, and possibly, again between the G1 and G2 generations. The "stable germ line transformation" claimed (p.83) is based on a dangerous instability of the insert, which is prone to secondary mobilisation.

The proposal for the field test is based on the belief that the *piggyBac* gene inserts would be stable. But there is already evidence, described above, that they are not stable. Furthermore, *piggyBac* may be carried by the baculovirus that infect insects, and such virus certainly produces transposase that can move the gene-bearing transposon. The virus can also act as a vector for rapid spread of the modified transposon to a variety of insects.

The proposal claims that no human health concerns are involved in the field trail. It argues "Lepidoptera, in general, do not pose a threat to human health and welfare and should remain a guiding principle in deciding on human risks related to their genetic manipulation." However, the same *piggyBac* transposon was also used for gene transfer in the mosquito that transmits yellow fever. Worse still, baculovirus, which harbours *piggyBac* transposon, is used in human gene therapy because it is so good at getting into human cells, so any *piggyBac* it carries will be efficiently smuggled along.

These artificial transposons are already aggressive and promiscuous genome invaders, and putting them into insects is to give them wings, as well as sharp mouthparts for efficient delivery to all plants and animals and their viruses. The predictable result is rampant horizontal gene transfer and recombination across species barriers. The unpredictable unknown is what kinds of new deadly viruses might be generated [5], and how many new cases of insertion mutagenesis and carcinogenesis they may bring [6]. It is the height of folly and irresponsibility to release such GM insects, let alone GM insects carrying female-killing genes (see "Terminator insects – the killing of females", this issue).

There is a compelling case for stopping these developments altogether on grounds of hazards that can already be foreseen.


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2. See "Terminator insects – a primer" by Joe Cummins, ISIS Report, March 2001 www.i-sis.org
3. Lobo N, Li X and Fraser Jr. MJ. Transposition of the *piggyBac* element in embryos of Dro*sophila melanogaster*, *Aedes aegypti* and *Trichoplusia ni*. *Mol Gen Genet* 1999: 261: 803-10.
4. Toshiki T, Chantal T, Corinne R, Toshio K, et al. Germline transformation of the silkworm *Bombyx mori L.* using a *piggyBac* transposon-derived vector. *Nature Biotechnology* 2000: 18: 81-84.
5. See "Genetic engineering superviruses" by Mae-Wan Ho, ISIS Report, March 2001 www.i-sis.org
6. See "Unregulated hazards, 'naked' and 'free' nucleic acids" ISIS Report, Jan. 2000 www.i-sis.org


## Terminator Insects – The Killing of Females

*GM pink bollworms are a prelude to developing female-killing traits to control bollworm pests.* ***Dr. Mae-Wan Ho*** *and* ***Prof. Joe Cummins*** *explain the genetics and hazards of female killing systems.*

The female-killing systems developed out of the sterile insect technique (SIT), which has been used in biological pest control since the 1950s. It involves mass rearing and release of insects made sterile with X-irradiation and other methods. Initially, sterile insects of both sexes were released, but sterile females were thought to be detrimental to pest control. One main reason is

that a single male can mate with a large number of females, while each female will mate with only a few males. Hence, genetic sorting mechanisms (GSMs) were invented to kill the females. Until quite recently, all GSMs have involved radiation-induced X-Y translocation, ie moving of part of a normal X chromosome to the Y chromosome. The resultant Y chromosome then acts as a dominant selectable marker in a population in which all the X chromosomes carry a gene that is 'conditional lethal' in double dose. A conditional lethal is a gene that kills only under certain 'non-permissive' conditions, as for example, exposure to heat. Thus, when the population is heat shocked, the males (XY) survive, while the females (XX) die.

Recently, other conditional lethal systems have been considered for kill off females. Such systems could be introduced into any insect pest species with the help of genetic engineering. As a result, the GM insects could be released directly without pre-sterilisation. One method involves creating a strain that carries a conditional, sex-specific lethal gene, ie, a lethal gene that is expressed only in one sex under 'non-permissive' conditions. The design is such that the non-permissive condition is one that is normally found in nature, whereas the permissive condition (one that permits survival) depends on certain chemicals that could be added to the diet in the insect factory. Researchers have constructed such a system in *Drosophila*.

They make use of special transcription control elements (ie promoters) and transcription factors, proteins that bind to promoters to enhance transcription [1]. First, the transcription factor, tTa, a protein that interacts with tetracycline, is placed under the control of a promoter, *Yp3*, which is active in female larvae and adults, but not in males. Next, a reporter gene lacZ, coding for β-galactosidase is placed under another promoter, the tetracycline responsive element, *tRe*. In the absence of tetracycline, tTa binds to *tRe* causing the reporter gene to become expressed. In the presence of tetracycline, however, the tetracycline binds to tTa, thereby preventing it from binding to *tRe*, and the reporter gene is not expressed. (Note: the convention is that genes are in italics, whereas the corresponding gene products are non-italics.)

Strains of flies homozygous for the constructs, *Yp3-tTa* and *tRe-lacZ*, respectively were crossed with each other. The resulting progeny were raised in the presence and absence of tetracycline in the culture medium. Adults were stained for β-galactosidase activity. Females grown on normal diet without tetracycline stained strongly for the enzyme, whereas females raised on tetracycline and all males were negative.

To engineer the killing of females, a toxic gene product, Ras64B, was placed under the control of *tRe*; and a line with *tRe-Ras64B* was constructed. (*Ras1* is a gene that codes for a protein that plays a key role in regulating transcription in the cell. *Ras 64B* is a defective mutant allele of *Ras1*. *Ras* homologues are oncogenes contributing to human cancer.) The *tRe-Ras64B* line was crossed with another line in which the *tTa* was placed under a nonspecific (constitutive) promoter. The progeny grown on tetracycline were viable and fertile. On normal medium, however, no progeny survived, ie, both males and females died. When the *tRe-Ras64B* line was crossed to the *Yp3-tTa* line, the male progeny but not the female survived in the absence of tetracycline.

Subsequently, the researchers constructed another line homozygous for both *Yp3-tTa* and *tRe-Ras64B* on the same chromosome, which was maintained on medium with tetracycline to inhibit the expression of *Ras64B*. When the flies were transferred to medium without tetracycline, no female progeny were recovered in a sample of more than 5000 males. The genetic system also worked with gene products that are specifically toxic to females.

The males were fertile when mated to other females. This is important for spreading the female-killing gene throughout the pest population. However, it would also spread the gene to related species. The potential also exists for horizontal transfer to unrelated species.

The proposal to engineer these genes into promiscuous transposon vectors will greatly multiply the risks of horizontal transfer to unrelated species, with potentially disastrous effects on biodiversity through the killing of females.

1. Thomas DD, Donnelly CA, Wood RJ and Alphey LS. Insect population control using a dominant, repressible, lethal genetic system. *Science* 2000: 287: 2474-6.

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## Terminator Patents Decoded

*Terminator technology is a collection of genetic engineering tricks to make seeds sterile, so farmers cannot save and replant the seeds. The sole purpose of this technology, now owned by the big seed corporations in collusion with the US government, is to control seed production at source. It violates the basic human right of people to grow their food from saved seeds, and also introduce some of the most dangerous genes and constructs into crop-plants. This highly immoral and*

*hazardous development must be stopped. All terminator crops that have been released commercially or undergoing field trials must be recalled and destroyed.*
*ISIS exposed the duplicity of biotech corporations that have been testing and growing terminator crops since 1990, while pretending that none has yet been produced.* ***Dr. Mae-Wan Ho*** *and* ***Prof. Joe Cummins*** *have written a primer explaining the technology in general terms (see "Terminator crops are here, ISIS exclusive" and "Killing fields near You", ISIS News 7/8). This sequel article unravels several different versions of the technology that have been patented.*
The patents cover not only terminator techniques that engineer seed/pollen sterility, but also the control of expression of specific traits such as insect tolerance, drought tolerance or modification of secondary metabolism.

The overall aim is certainly to control either seed production or agronomically important traits at source. The genes used, as well as the constructs will have catastrophic consequences on biodiversity and health.

**USDA and Delta and Pineland Company patent US5925808: Control of Plant Gene Expression**, filed July 20, 1999/ Dec. 19, 1997
This is substantially the same as US 5723765, granted in 1998. It is a broad patent that includes not only constructs for controlling plant gene expression, but also the methods whereby the transgenic plants are generated by transformation, the vectors for transformation, and the various crosses between plants. Cotton plants are mentioned specifically in this patent.
The main constructs are as follows,
1. A lethal, terminator gene, call it *gene a,* linked to a transiently active promoter, call it *p*(*t*), the gene and promoter being separated by a blocking sequence, call it *block* flanked on either side by specific excision sequences, call them *ex*.

*p(t)*-*ex*-*block*-*ex*-*gene a*

2. A second gene, call it *recom*, encoding a recombinase, specific for the excision sequence *ex* of the first construct, linked to a repressible promoter, call it *p(r)*, that is active during seed germination.

*p(r)*-*recom*

3. A third gene, call it *repress*, encoding the repressor that binds to the repressible promoter *p(r)* to turn the second gene off. Although not mentioned in this patent, the repressor is one that can respond to an external chemical, such as tetracycline, which through a tetracycline responsive promoter *p(tet)* linked to the repressor, can turn the repressor on (or off, in another version).

*p(tet)*-*repress*

In one version of how this is intended to work, the seeds are germinated, by the company, in the presence of tetracycline, which turns on the *repress* gene, the repressor protein binds to *p(r)* and stops *recom* from being expressed, so *gene a* is blocked, and nothing happens.

In the absence of tetracycline, say, when the farmer sows the seeds, the repressor protein is not expressed, so during seed germination, *recom* is turned on to make recombinase. The recombinase snips out the blocking sequence, *block*, and *gene a* is expressed. If *gene a* is a lethal gene that kills the male part of the flower, and *p(t)* is a promoter that acts only in the male part of the flower, the plant will be male sterile. If *gene a* and its promoter are specific for the female part of the flower, the plant will be female sterile. If *gene a* and its promoter are specific for germination, then seeds will set, but they can't germinate.

Each of the genes, promoters and repressors itself can be any one selected from an entire group of possibilities.

Thus, *gene a* may be one of the following: lethal genes that kill the cell (terminator gene proper), insecticidal gene, fungistatic gene, fungicidal gene, bacteriocidal gene, drought resistance gene, protein gene product or a gene that alters secondary metabolism.
Similarly, the transiently active promoter *p(t)* may be a promoter that is active in late embryogenesis, in seed development, in flower development, leaf development, root development vascular tissue development, pollen development (male sterility), after wounding, during hot and cold stress, water stress, or during or after exposure to heavy metal.

The specific excision sequences and recombinase are selected from a group that comprise not only site-specific recombinase but transposase, flippase, resolvase, and integrase,
Male sterility includes any lethal gene linked to an anther-specific promoter or pollen-specific promoter. Lethal genes include ribosomal inhibitor protein.

A lethal gene linked to a promoter that is active during late embryogenesis, for example, will give rise to seeds that are sterile, one that is linked to a promoter active during germination will result in seeds that fail to germinate.
The blocking sequence can be a sequence that confers male sterility.

**Syngenta (Zeneca) patent US 5808034: Plant gene construct comprising male flower specific promote[r],** filed 15 September 1998).
This is an update on a patent first filed in 1990. It involves a cascade of gene regulation, the end result is the expression of a protein that disrupts pollen development. The disrupter protein is restricted to the male parts of the plant by an upstream promoter specific to male flowers. The male specific promoter being placed under the control of a regulatory sequence called the *operator*, that is turned off by a repressor protein binding specifically to it, and the expression of the repressor protein can be induced by a specific chemical externally applied.
The constructs are as follows:
1. A promoter *p(l)* responsive to the presence or absence of an exogenous chemical inducer, linked to the gene *repress* for the repressor protein.

*p(l)*-*repress*

2. An operator *op* responsive to the repressor protein linked to the male specific promoter *p(m),* which is linked in turn to *disrupt*, the gene for the disrupter protein that kills the pollen.

*op*-*p(m)*-*disrupt*

When the chemical inducer is applied, the cascade goes as follows:
external chemical inducer ➞ repressor ➞ operator ➞ no expression of disrupter protein.
The net result is the line can be maintained.

In the absence of the external chemical inducer, the repressor is not expressed, so the disrupter protein is expressed and the result is male sterility.
This is rather similar to the USDA-Delta Pine patent above, but the constructs are a bit simpler, and do not involve a recombinase.

Clearly the company can sell the proprietary chemical inducer to restore fertility to the line or to maintain it. As said, the whole point of the patent is to control the production of fertile seeds.
As in the USDA-Delta Pine patent above, each element in the patent can be realised with any one from a whole group of possibilities.
The chemical switch, *p(l)*, is exemplified by the promoter of the maize glutathione-S-transferase (GST II) gene, which is responsive to a host of chemicals, called ‘safeners’. Safeners, also known as antidotes are used to protect crops from herbicide injury, as they induce a family of enzymes, glutathione-S-transferases which catalyse the detoxification of a large range of hydrophobic (water-insoluble, fat-soluble) electrophilic (eletron-loving) compounds, ie herbicides, by joining up with them via the sulphydryl group, and causing their removal from the body of insects and mammals.

The patent lists many potential chemical inducers of the GST II gene . Safeners are used in combination with herbicides to reduce crop damage from the herbicide. The herbicide families requiring safeners are thiocarbamate and chloroacetanilide herbicides used to control weeds in corn, rice, sorgum and other grasses.

The chemicals listed in the patents include the safteners with common names like flurazole, napthinic anhydride, dicyclonon, oxabentrinil, fenclorim, cyometril, fluxofenim, furilazole and dietholate. There do not seem to be many publications reporting on the safety of the safeners.

**United States Patent 5,750,867(Plant Genetic Systems, now Aventis): Maintenance of male-sterile plants**, filed May 12, 1998
This patent, first filed in 1992, covers “transgenic plants that have, *stably* integrated into their nuclear genome, a maintainer gene comprising a fertility-restorer gene and a pollen-lethality gene” (italics ours, because we don’t believe any evidence exists that the integrated foreign DNA is indeed stable). The plants can be used to maintain a homogeneous population of male-sterile

plants. This specific patent covers maize plants, but the method has already been tried out in oilseed rape.

A complicated process for maintaining a male-sterile line is required, in which a male-sterile line is crossed with a 'maintainer line'. The male-sterile line is homozygous for a male-sterile gene, *barnase*, coding for a ribonuclease (barnase) from the bacterium *Bacillus amyloliguefaciens*, placed under an anther-specific promoter *p(a)*, *which acts early in the development of the male flower*. In other words, it has two copies of the construct, one on each of a pair of chromosomes. This can be represented as follows.

*p(a)*-*barnase*

*p(a)*-*barnase*

The 'maintainer line' is male fertile. It has the same genotype as the male-sterile line (ie, it is homozygous for the male sterile gene and stamen-specific promoter), and in addition, and not linked with the male-sterility gene, is heterozygous for a 'restorer gene' directed by a 'restorer promoter', *p(r)*, which is at least also expressed in the stamen cells, linked with a 'pollen-lethality' gene under the control of a pollen-specific promoter *p(m)*, ie, *one which is expressed late in the development of the male flower, in pollen-cells after* meiosis, the cell division leading to halving the chromosome complement. (The male-sterility gene is expressed *before* meiosis.) The restorer gene, *barstar* producing the protein barstar, also from *Bacillus amyloliguefaciens*, is a specific inhibitor of the male sterility gene product, barnase, while the 'pollen-lethality' gene, *lethal*, prevents pollen from being formed. The genotype of the maintainer line can be represented as follows (the big rectangle on the outside represents a cell).

***Before meiosis***

*p(a)*-*barnase* *p(r)*-*barstar*-*p(m)*-*lethal*

*p(a)*-*barnase* -

***After meiosis***

*p(a)*-*barnase* *p(r)*-*barstar*-*p(m)*-*lethal*

*p(a)*-*barnase* -

As can be seen, after meiosis, the only viable pollen is the one with barnase, which confers male sterility. This pollen will spread the male-sterility trait around.

Each piece of the genetic jigsaw can be any one of several genes. Thus, *p(a)* can be the promoter of the *zm 13* gene from maize or the *TA29* gene from tobacco, or any promoter that directs expression in the tapetum cells of the stamen. The male-sterility gene *barnase*, can be any ribonuclease instead of barnase, while the restorer gene could be any ribonuclease-inhibitor instead of barstar, which is specific for barnase. The restorer promoter *p(r)* could be identical to *p(a)* so long as it leads to the expression of the restorer protein *at the same time and in the same cells* as the terminator protein.

As the restorer gene and the sterility gene are not linked, half of the ovule (female part) of the restorer plants, on average, will be male-sterile with barnase only. The other half will have both barnase and the barstar linked to the pollen-lethal gene. The pollen-lethal gene linked to the restorer gene prevents the male gametes containing the restorer gene from developing, so the only pollen available is one without the restorer gene, but carrying the male-sterility gene all the same (see diagram above).

When the maintainer line is selfed, the progeny will consist of 50% heterozygotes, ie, same as the maintainner line, and 50% male-sterile. So, it is necessary to incorporate a selectable marker, such as herbicide tolerance, next to the male-sterile gene or the restorer gene, or a different selectable marker can be put next to each. Say, a gene coding for phosphinothricin acetyl

transferase (PAT) linked to the barnase gene in the male-sterile line, and that will enable only male-sterile seeds to be selected.

**United States Patent 5,633,441 (Plant Genetic Systems, now Aventis): Plants with genetic female sterility** filed May 27, 1997,

This patent, first filed in 1990, is similar to the male-sterile patent, except that a female-specific promoter is used to control expression of a lethal terminator gene in the female part of the flower without affecting the male part. The female-sterility gene is linked to a selectable marker gene with its own promoter, so that the female-sterile plants can be selected. In addition, a 'transit-peptide' is included in both the female-sterility gene and the marker gene to direct the gene product into chloroplasts or mitochondria, presumably so it does not affect pollen development, although many plants do have chloroplasts in pollen.

Terminator genes include, besides barnase, papain active protein, or the A-fragment of diphtheria toxin. Marker genes used include herbicide resistance gene, or a gene conferring a disease or pest resistance, a GUS gene for glucuronidase, or a gene encoding a *Bacillus thuringiensis* (Bt) endotoxin.

The second promoter, linked to the marker gene, may be a constitutive promoter (expression at all times in all cells), a wound-inducible promoter, a promoter which directs gene expression selectively in plant tissue having photosynthetic activity, or a promoter which directs gene expression selectively in leaf cells, petal cells or seed cells.

The patent claims include methods and vectors for making the transgenic plants, the various bits of DNA, the genes as well as the style-, stigma-, ovary-, seed- and embryo-specific promoters. Also claimed are the cell cultures, the hybrid seeds produced by crossing the female-sterile plant with a female-fertile plant; and a process for producing such hybrid seeds, as well as seedless fruit.

The plants for which the patent is claimed include corn, potato, tomato, oilseed rape or other Brassica species, alfalfa, sunflower, cotton, celery, soybean, tobacco, and sugarbeet.

**Hazards galore**

We have pointed out the hazards of terminator technology in earlier papers ("Terminator in new guises", *ISIS News* 3, December, 1999; "Killing fields near you", *ISIS News*7/8 Feb. 2001), and will only briefly recapitulate them here.

There are many different constructs, all of which have to be precisely engineered, and integrated into plants as intended, which is beyond the capability of current technology. A lot of gene scrambling occurs in artificial GM constructs as they are integrated, and genetic engineers cannot control where they are integrated either, thus multiplying the uncertainties and unpredictability of the GM crops produced.

The recombinase and similar enzymes in the USDA-Delta Pine patent is perhaps the most dangerous, as it is known to cause recombination at non-specific sites, thereby causing largescale genome scrambling (see "Terminator recombinase does scramble genomes", *ISIS News* 7/8).

The terminator lethal genes and gene products are known to be harmful to cells, including mammalian cells. Some of the most hazardous genes are designed to spread through pollen, including 'male-sterile' gene-constructs, and indeed, female-sterile constructs.

Genes and GM constructs can spread, not just through cross-pollination, but by horizontal gene transfer to unrelated species, and this process cannot be controlled. The instability of GM constructs in general and the complicated ones in terminator constructs in particular, increase the propensity for horizontal gene transfer and recombination. Horizontal gene transfer and recombination is one of the main routes for generating new viruses and bacteria that cause diseases, and for spreading drug and antibiotic resistance to make the diseases untreatable.

***This highly hazardous and immoral development must be stopped, and all terminator crops that have been released commercially or undergoing field trials must be recalled and destroyed***.