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REVIEW

Toward two decades of plant biotechnology: successes, failures, and prospects

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Abstract

The use of plant biotechnology in agriculture approaches the end of its second decade. While it is now a maturing industry in the Americas, Asia, and Australasia in particular, in some parts of the world, no more so than in Europe, it remains a highly controversial issue. European authorities have responded to the controversy by establishing a regulatory framework so impenetrable that development of the technology in Europe has effectively ground to a halt and the United Kingdom, for example, is no nearer to the commercial cultivation of genetically modified (GM) crops than it was when the first GM varieties went on the market in the United States of America in the mid-1990s. This review covers the GM crop varieties and traits that have been launched in the last 18 years, including the failures as well as successes, and considers the prospects for the technology.

History

Modern, scientific plant breeding arose from the rediscovery of Mendel's 1866 work, "Versuche uber Pflanzen-Hybride," in 1900. The "Russett Burbank" hybrid potato (*Solanum tuberosum*) variety was launched in 1923 and the first hybrid maize (*Zea mays*) variety was not released until 1933. By the 1950s, scientists were using chemical and radiation mutagenesis to increase the genetic variation from which they could breed new varieties, and in the 1960s and 1970s, thanks to the inspiration of Norman Borlaug, dwarfing genes were incorporated into breeding programs worldwide, bringing about the "Green Revolution" and averting a global food crisis.

Advances in molecular biology were being made rapidly at this time and the first recombinant DNA molecule was reported by Berg and coworkers in 1972 (Jackson et al. 1972). Five years later, in 1977, Chilton and coworkers described the natural genetic modification of host plant cells by *Agrobacterium tumefaciens* (Chilton et al. 1977) and only 6 years after that, in 1983, Hall reported the production of genetically modified (GM) sunflower (*Helianthus annuus*) plants containing a gene from bean (*Phaseolus vulgaris*) (Murai et al. 1983). Eleven years on, in 1994, a U.S. company, Calgene, launched a GM tomato (*Lycopersicon esculentum*) variety "Flavr Savr" and by 1996 the first significant areas of GM commodity crops, soybean (*Glycine max*) and maize, were being grown, and the genie was irrefutably out of the bottle.

Definitions

At this point, it would be useful to define what is meant by GM. New varieties of crops are usually marketed with claims of improved traits of one sort or another compared with currently available varieties, and traits are

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genetically controlled. In the United Kingdom, new varieties of agricultural and vegetable species must be placed on a National List to be eligible for certification and marketing. To be added to the National List, a variety must be "distinct, sufficiently uniform, and stable (DUS)" and, for agricultural crops, "have satisfactory value for cultivation and use (VCU)" (http://www.fera.defra.gov.uk/plants/plantVarieties/nationalListing/). This is a legal requirement and a new variety will only be added to the list if it is genuinely new and an improvement on varieties already available. Essentially, this means that a new variety must be genetically different from any other variety already on the market. However, it would not be described as GM: that term has come to be used specifically to describe a plant or variety that contains a gene or genes that have been introduced artificially, and no varieties of that sort are currently being marketed in the United Kingdom. Such plants are also described as being transgenic, having been transformed, or as genetically engineered (GE).

Genetic modification is therefore a term that is based on the technique that is used to produce a GM plant, not on the nature of the plant. In that respect, it is somewhat unsatisfactory because technology continues to move on. Several methods already available to the plant biotechnologist use the common soil bacterium, *Agrobacterium tumefaciens,* which will infect wounded plant tissue and insert a short section of DNA, called the transfer DNA or T-DNA, into the host plant genome (Chilton et al. 1977). *A. tumefaciens* can be used through the infection of explants or protoplasts and the regeneration of GM plants from tissue-cultured transformed cells. Methods have also been developed that do not require tissue culture, such as floral dip (Bechtold et al. 1993; Clough and Bent 1998), in which plants at the early stages of flowering are placed in a suspension of *A. tumefaciens* in a vacuum jar, a vacuum is applied to remove air surrounding the plant tissue and allow the bacteria to come into contact with the plant cells, and the plants are grown to seed. Typically, approximately 1% of the seeds are genetically modified. This method is now widely used in basic research using Arabidopsis (*Arabidopsis thaliana*) and has been adapted with some success for use with other plant species, including soybean and rice (*Oryza sativa*).

Protoplasts can also be induced to take up DNA directly, either by treatment with polyethylene glycol (PEG) or by electroporation. This process is called direct or DNA-mediated gene transfer and in a small proportion of the protoplasts, the introduced DNA will integrate into the host DNA and the protoplast will be stably transformed. The protoplast can then be induced to form callus, from which a GM plant can be regenerated. Electroporation can be applied to intact cells in tissue pieces or in suspension, as well as to protoplasts, but this has only been shown to work efficiently in a few species.

Another direct gene transfer method to have been developed is silicon carbide fiber vortexing. Plant cells are suspended in a medium containing DNA and microscopic silicon carbide fibers. The suspension is vortexed and the fibers penetrate the plant cell walls, allowing the DNA to enter. Finally, there is particle bombardment, in which plant cells are bombarded with tiny particles coated with DNA. Particle bombardment has been particularly successful in the production of GM cereals and the subculturing of shoots of GM wheat (*Triticum aestivum*) forming from callus derived from the particle bombardment of embryos is shown in Figure 1. However, it has the disadvantage that the GM plant that is produced is often found to have multiple copies of the transgene. This makes genetic analysis of the GM plant and the production of a homozygous line from it more difficult. It has also been a problem in obtaining permission for marketing in Europe, where regulators expect to receive detailed information on all of the sites where the transgene has been inserted. The method was developed in part because *A. tumefaciens*-mediated transformation of cereals used to be very difficult. Strains of the bacterium have now been developed that infect and transform cereals much more efficiently and this is probably going to be the preferred option in cereal biotechnology in the future.

In recent years, commentators have begun to use the terms first and second generation to describe commercial GM crop applications that involve input traits and output traits, respectively. Input traits affect the husbandry and management of a crop, and include, for example, herbicide tolerance, resistance to insects or pathogens, and the ability to survive stress conditions, such as drought. Farmers are the principal beneficiaries, although consumers may benefit indirectly through lower food prices.

Figure 1. Subculturing shoots forming from callus derived from particle bombardment of wheat (*Triticum aestivum*) embryos. Picture kindly provided by Rothamsted Research.

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Output traits affect the composition of the crop product and include, for example, changes in the fatty acid composition of oils, changes in starch quality, improved nutritional value, for example, through increased vitamin content, or better processing properties. The main beneficiaries are consumers and/or food processors.

GM varieties with improved input traits have undoubtedly been easier to sell to farmers, and farmers are the customers of seed companies, not food processors, retailers or consumers. Some of these varieties are now well established and have been extremely successful, while varieties with improved output traits have been relatively marginal. The terms first and second generation have been used to highlight the fact that more GM varieties with improved output traits are expected to come to the market in the near future. However, they reflect a simplistic view, firstly because the first GM variety on the market back in 1994 was the "FlavrSavr" tomato, a variety with improved shelf life (an output trait); secondly, because output traits are likely to be combined with (first generation) input traits, providing added value to varieties with traits that farmers are already familiar with and want.

New Technologies

Genetic modification of plants is now almost three decades old. Not surprisingly, technology has moved on, and there are a number of new techniques that may or may not come under the GM banner and, therefore, GM regulations. Indeed, the European Commission set up a working group in December 2008 to decide how these new techniques should be regulated. The techniques include the following:

Cisgenesis (sometimes called intragenesis)

This is not really a new technique, it simply refers to genetic modification using one of the techniques described above, but using no "foreign" DNA; in other words, the manipulation is done using DNA entirely from the same species as the host plant, or a species that is closely related enough to be sexually compatible. The use of the term is an attempt to distinguish GM plants or other organisms produced in this way from transgenics, that is GM plants that contain DNA from unrelated organisms. It has been argued that cisgenic GM plants should not be regulated in the same way as transgenic GM plants (Schouten et al. 2006), and the Symplot company of the U.S.A., for example, has used what it called "all-native" DNA to transform potatoes to reduce acrylamide formation during processing (Rommens et al. 2006). It has proven difficult to establish a market for GM potatoes even in the United States of America and Symplot may be trying to allay fears by using this technique. However, it is not clear yet whether the strategy will be successful in the United States of America and it seems unlikely that cisgenics will be treated any differently to transgenics in Europe.

Zinc-finger nuclease technology

Zinc-finger nucleases are artificial enzymes produced by fusing a zinc-finger binding domain to the nonspecific DNA cleavage domain of a restriction enzyme, usually that of FokI. The zinc-finger domain can be engineered to target a specific nucleotide sequence, and the cleavage domain must dimerize to work, so two nucleases are required, one to bind to each strand of the DNA, increasing the specificity. The host cell's own enzymes repair the DNA, resulting in highly specific, targeted alterations to the DNA sequence; a form of targeted mutagenesis. The technique has been demonstrated to work in tobacco (*Nicotiana tabacum*) protoplasts (Townsend et al. 2009).

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODGM) is another technique devised to introduce specific mutations at defined sites of the genome. It is based on site-specific mutation of a target gene by the introduction of an oligonucleotide with an identical nucleotide sequence to the target gene apart from the nucleotide to be substituted. The oligonucleotide, usually between 20 and 100 nucleotides in length, is delivered by electroporation or PEG-mediated transfection of protoplasts, and interacts with the target gene. It does not actually insert into the genome; rather, as with the zinc-finger nuclease technique, the mutation is incorporated into the genomic DNA by the native DNA repair machinery. The technique has been successful using single-stranded DNA oligonucleotides, RNA oligonucleotides, or chimeric oligonucleotides consisting of both RNA and single-stranded DNA. Triple helix-forming oligonucleotides (TFOs) have also been used; a triple helix can form when a TFO binds in a sequence-specific manner in the major groove of duplex DNA. The technique therefore has a variety of alternative names, depending on the type of oligonucleotide used, the delivery method and the specific application. These include targeted nucleotide exchange, chimeraplasty, oligonucleotide-mediated gene editing, chimeric oligonucleotide-dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotide-induced recombination, therapeutic nucleic acid repair, and targeted gene repair.

ODGM has been used successfully in plants (Beetham et al. 1999; Zhu et al. 1999; Kochevenko and Willmitzer

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2003) and the U.K.'s Advisory Committee for Releases to the Environment was asked for an opinion on an oilseed rape variety developed by Cibus Global that could tolerate sulfonylurea herbicides as a result of a mutation introduced by ODGM. The Committee concluded that organisms produced by mutagenesis could be excluded from European Union (EU) directives covering GM organisms as long as they did not contain recombinant nucleic acid molecules (http://www.defra.gov.uk/acre/files/20110319-Cibus-advice.pdf, 7 March 2011). The United States Department of Agriculture reached a similar conclusion, but the European Food Safety Authority (EFSA) has not yet delivered its opinion on the matter.

RNA-dependent DNA methylation

RNA interference is an established GM technique that exploits natural mechanisms of RNA silencing (reviewed by Baulcombe 2004). A plant is modified to synthesize a double-stranded RNA molecule derived from a target gene, for example, by using a gene construct in which part of the gene is spliced sequentially in a head-to-tail formation downstream of a single promoter. This causes the production of an RNA molecule that forms a hairpin loop (hpRNA); this molecule is cleaved into short, double-stranded RNA molecules by an enzyme called Dicer that is naturally present in the cell. These short RNA molecules are called short interfering RNAs (siRNAs). The siRNAs are unwound into two single-stranded molecules, one of which (the passenger strand) is degraded, while the other (the guide strand) is incorporated into a RNA-induced silencing complex (RISC). The guide strand pairs with the complementary sequence of messenger RNA from the target gene, and induces cleavage by another enzyme, Argonaute, which is present in the RISC.

Plants also use double-stranded RNAs (dsRNAs) to induce cytosine methylation of DNA, leading to the formation of transcriptionally silent heterochromatin. The RNAs that trigger DNA methylation can come from a variety of sources, including viruses and transposons. However, promoter methylation and transcriptional silencing has been demonstrated in plants as a result of dsRNA arising from a transgene (Mette et al. 2000).

Agro-infiltration

In this method, transient expression of a gene is achieved by introducing a suspension of *A. tumefaciens* to the underside of a plant leaf, usually via a syringe without a needle. The *Agrobacterium* enters the air spaces within the leaf via the stomata and delivers the transgene to some of the leaf cells. Alternatively, leaves, leaf disks or even whole plants are placed in a beaker containing the *Agrobacterium* suspension. The beaker is then placed in a vacuum chamber and a vacuum applied, forcing air out of the stomata. When the vacuum is released, the bacteria are drawn deep into the leaf tissue. Either way, the plant is not stably transformed and the transgene is not inherited, so while this technique will no doubt be useful in the study of plant gene function, it has little relevance to commercial agriculture.

Why Use GM?

Plant breeding has been extremely successful; average U.K. wheat yield at the turn of the 20th century, for example, was around two tonnes per hectare, whereas it is now around four times that. While mechanization and the development of artificial fertilizers, pesticides, and herbicides have contributed significantly to that improvement, plant breeding has also played an important part. This spectacular improvement has been achieved without the use of biotechnology, and the genetic information and resources available to assist in non-GM breeding in the genomics era greatly exceed what previous generations of plant breeders had to work with. So do we need GM at all?

The answer to that question is that GM enables plant breeders to do some things that would not be possible by other methods. GM is an additional tool in the plant breeder's box; not sweeping everything else away and not a panacea that is applicable to every plant breeding program, but nevertheless, a powerful technique when applied to an appropriate target. For example, it allows genes to be introduced into a crop plant from any source: sexual compatibility is not required. It is relatively precise, in that single genes can be transferred, and genes can be designed to be active at different stages of a plant's development or in specific organs, tissues or cell types, something that is not possible by any other method. A gene can also be altered before being engineered into the host plant to change the properties of the protein that it encodes, and the nature and properties of the protein can be studied to ensure that the gene is safe to use.

GM also has some disadvantages. A successful GM program requires background knowledge of a gene, the protein that it encodes, and the other genes and proteins that interact with it. This requires a significant investment of time and money compared, for example, with the generation of random mutations. Most significantly, however, GM varieties have to undergo much more detailed analysis and testing than new non-GM varieties, particularly in Europe, and this incurs a substantial cost. Anecdotal reports from the plant biotechnology industry put the

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estimated cost of developing a new GM variety at $100 million. This sort of investment is beyond the capability of most plant breeding organizations and represents a considerable risk for any company. Historically, regulations covering new technologies are gradually relaxed as the technology is shown to be safe to use, and the costs of regulatory compliance reduce. However, there is no sign of that happening with plant biotechnology, at least in Europe, and the need for producers to have access to the huge European food market means that obtaining permission to sell a new GM variety in Europe is essential in most cases. European attitudes to GM and the resulting regulations therefore affect the development of new GM varieties and traits throughout the world, and there are still only a handful of GM traits that have been used successfully in commercial agriculture. These are described in the following section.

Traits

Herbicide tolerance

Herbicide tolerance, along with insect resistance, took commercial plant biotechnology into global commodity crops and it remains the most successful and widely used GM crop trait. The strategy is a simple one: genetically modify a crop plant to tolerate a broad range herbicide; that is, a herbicide that kills any plant not carrying the tolerance gene. Most herbicides are selective in the types of plant that they kill and a farmer has to use a combination of herbicides that are tolerated by the crop, but kill the problem weeds. The herbicide regime may be complicated, some of the herbicides may only be effective pre-emergence, some may be toxic and difficult to handle safely, the equipment and labor required may be costly and the farmer has to be careful that herbicides applied one season do not persist to the next if a different crop is to be planted. Using GM crops that tolerate broad-range herbicides (GM-HT crops) eradicates many of these problems and not surprisingly, therefore, GM-HT varieties have been extremely popular wherever farmers have been allowed to use them. The first GM-HT variety to be introduced was a "Roundup-Ready" soybean, which was produced by Monsanto and has been marketed since 1996. Varieties carrying this trait now dominate global soybean production.

"Roundup" is Monsanto's trade name for glyphosate, a broad range herbicide that was introduced as a commercial product in 1974 and therefore had two decades of use by farmers and gardeners, primarily to clear fields or to remove weeds from pathways, prior to the development of GM-HT varieties. It is not persistent and is taken up through the foliage of a plant, so can be applied "over the top" after the GM-HT crop is established, if necessary. Its target is 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), the enzyme that catalyzes the formation of 5-enolpyruvoylshikimate 3-phosphate from phosphoenolpyruvate and shikimate 3-phosphate (Fig. 2). This reaction is the penultimate step in the shikimate pathway, which results in the formation of chorismate, which in turn is required for the synthesis of many aromatic plant metabolites, including the amino acids phenylalanine, tyrosine, and tryptophan. The shikimate pathway is not present in animals, making glyphosate relatively nontoxic to animals, including man, and glyphosate is regarded by farmers as one of the safest agrochemicals to use. The familiarity of glyphosate undoubtedly helped to persuade farmers to adopt glyphosate-tolerant GM varieties when the varieties were launched.

Genetic modification of soybean to tolerate glyphosate (Padgette et al. 1995) was achieved by introducing an *EPSPS* gene from *A. tumefaciens* under the control of a *Cauliflower mosaic virus* (CaMV) 35S RNA (*CaMV35S*) gene promoter. The bacterial EPSPS is not affected by glyphosate, so plants carrying the transgene continue to have a functional shikimate pathway even when their own EPSPS is inhibited by the herbicide.

Glyphosate tolerance has now been engineered into cotton (*Gossypium hirsutum*), oilseed rape (*Brassica napus*), maize, alfalfa (*Medicago sativa*), sugar beet and fodder beet (*Beta vulgaris*). Brookes and Barfoot (2011) estimate a global increase in farm income of over 21 billion U.S. dollars between 1996 and 2007 from glyphosate-tolerant soybean alone, but perhaps the most convincing endorsement of the technology comes from the fact that farmers have adopted glyphosate-tolerant varieties with enthusiasm wherever they have been allowed to. The take-up of glyphosate-tolerant soybean varieties after their introduction in 1996, for example, rose to well over half of all the soybeans planted in the United States of America within 5 years and by 2010, 75% of the global soybean crop, which covered 100 million hectares, was GM, making it difficult to source non-GM soybean. Anecdotally, farmers cite the following benefits:

- Simpler and more flexible weed control.
- Reduced herbicide costs.
- Easier crop rotation because glyphosate is degraded rapidly in the soil.
- The ability to switch to a conservation tillage system, reducing soil erosion and nitrate leaching.
- Peace of mind because weed problems late in the season can be dealt with if necessary.

The other GM-HT trait on the market at present is gluphosinate tolerance (Fig. 2). This technology was developed by Plant Genome Systems, which was subsequently bought out by Aventis, which in turn was

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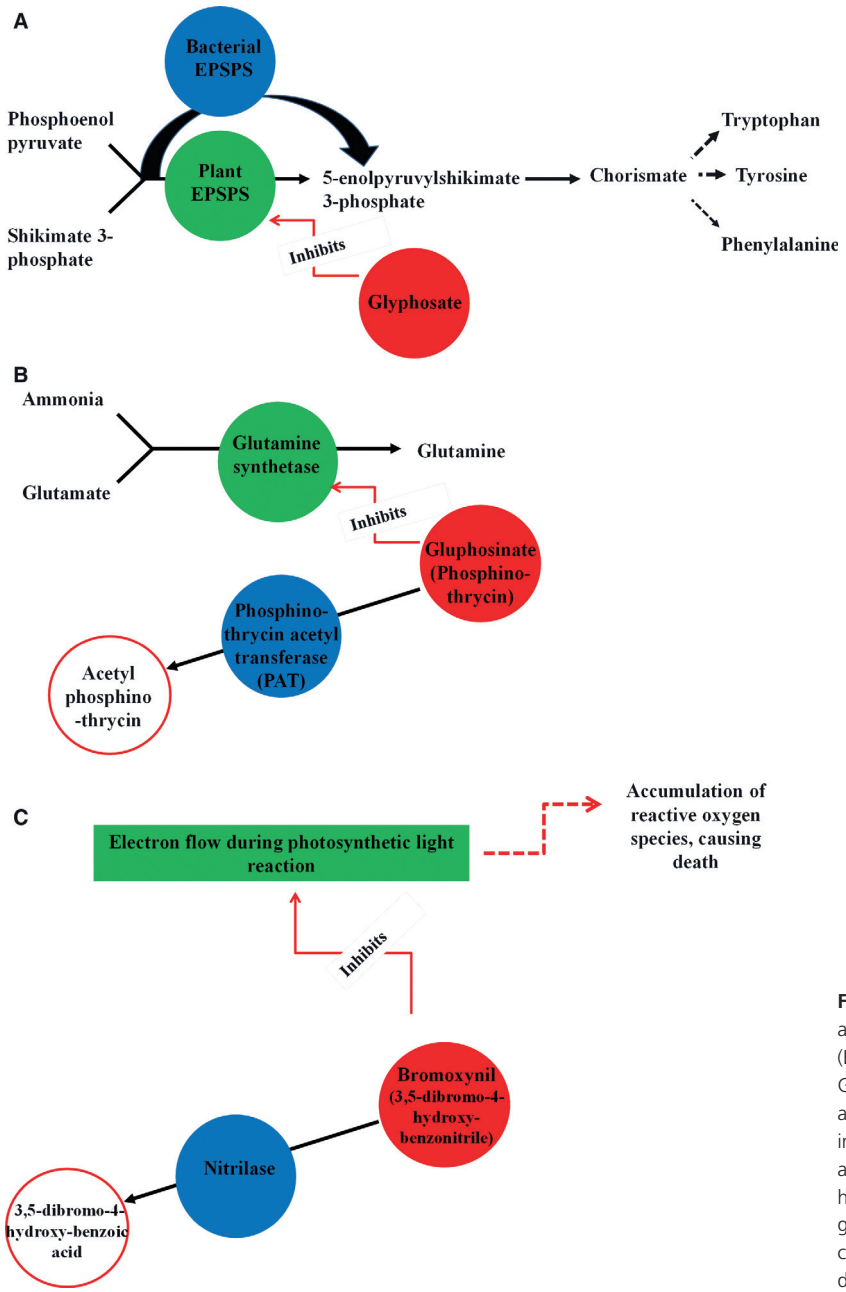

Figure 2. Diagrams illustrating the modes of action of broad range herbicides: (A) Glyphosate, (B) Gluphosinate, and (C) Bromoxynil; and the GM-HT strategies that have been devised to accompany them. Glyphosate tolerance is imparted by the introduction of a gene encoding an enzyme that effectively by-passes the herbicide, while the strategies for imparting gluphosinate and bromoxynil tolerance involve conversion of the herbicide into a nontoxic derivative.

acquired by Bayer. The gene that imparts tolerance to gluphosinate comes from the bacterium *Streptomyces hygroscopicus* and encodes phosphinothrycine acetyl transferase (PAT), which detoxifies the herbicide (de Block et al. 1987). The technology has been used in oilseed rape, maize, soybean, sugar beet, fodder beet, cotton, and rice. Bayer market gluphosinate under the trade name Liberty and varieties carrying the tolerance trait have the trade name LibertyLink.

Bayer also acquired the technology for a third GM-HT trait when they bought Aventis, the herbicides in this case being oxynil herbicides such as bromoxynil (3,5-dibromo-4-hydroxybenzonitrile). Bromoxynil and other oxynil herbicides inhibit photosynthesis by blocking electron flow during the light reaction, causing the production of reactive oxygen species (ROS), destruction of cell membranes, inhibition of chlorophyll formation and death. Tolerance is imparted by the *bxn* gene from bacterium *Klebsiella ozaenae*, which encodes a nitrilase enzyme that detoxifies the herbicide (Stalker and McBride 1987). This technology was used in an Aventis oilseed rape variety, Westar Oxy-235, which was marketed in Canada in the 1990s. However,

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Bayer withdrew Westar Oxy-235 in 2002 and there are currently no bromoxynil-tolerant varieties on the market.

The popularity of GM-HT varieties has raised fears that they could become victims of their own success, in that use of the same herbicide over large tracts of agricultural land for many years will mean that there is intense selective pressure on weeds to develop resistance, either independently or through acquisition of a tolerance gene through crossing with a GM-HT crop. The latter will, of course, depend on whether or not sexually compatible weed species are present where a GM-HT crop is being grown. There have been reports of the appearance of weeds that are poorly controlled by glyphosate (Owen 2008), and if left unchecked, the emergence of such weeds could threaten the use of GM-HT traits. Strategies being developed to preserve the effectiveness of GM-HT technology include engineering crops with additional tolerance traits, and Monsanto, for example, has announced that it intends to launch a soybean variety in which glyphosate tolerance is stacked with a trait imparting tolerance to dicamba (3,6-dichloro-2-methoxybenzoic acid), a pre-emergence herbicide (Behrens et al. 2007). Dicamba works by promoting plant growth to an unsustainable level so that the plant dies and is most effective against dicotyledonous (broadleaved) plants. The tolerance trait is imparted by a gene from a soil bacterium, *Pseudomonas maltophilia*, which encodes an enzyme that converts dicamba to 3,6-dichlorosalicylic acid (3,6-DCSA), a chemical with no herbicidal activity.

Insect resistance

The next most successful GM trait is insect resistance. Obviously, genetically modifying crop plants to reduce losses to insect grazing is highly desirable and many strategies have been tried and tested in the laboratory. Of these, only one has made it into commercial crop varieties, and that is the one based on the *Cry* genes of a soil bacterium, *Bacillus thuringiensis* (Bt). The *Cry* genes produce proteins that interfere with insect gut function. Different strains of the bacterium have different *Cry* genes, and these are classified into groups, *CryI–CryIV*, and subgroups, A, B, C etc. Each encodes a protein that is effective against a different type of insect: CryI proteins, for example, are effective against the larvae of butterflies and moths, while CryIII proteins are effective against beetles (reviewed by de Maagd et al. 1999).

As with glyphosate, Bt was already familiar to farmers before its use in plant biotechnology, in this case because it had been used as a pesticide for several decades, in the form of powders, granules, or aqueous, and oil-based liquids. Such "Bt" pesticides have a narrow host range and degrade rapidly, so they are not widely used in mainstream agriculture, but have been adopted by organic farmers as an "acceptable", biodegradable pesticide. They are also popular with salad farmers because they can be applied immediately before harvest due to their low toxicity to humans, as well as other mammals, birds, and fish.

The long use and excellent safety record of Bt pesticides, plus the fact that the active component was a protein encoded by a single gene, made the system an attractive one for crop biotechnology. Genetically modifying a crop plant to produce its own Cry protein overcomes the problem of rapid loss of activity after application of the conventional Bt insecticide. The most successful applications have been with the *Cry1A* gene, which has now been introduced into several crop species, including cotton, sugar beet, rice, soybean, and maize. GM varieties carrying the trait are usually referred to as Bt varieties. Their success in a particular area depends on the effectiveness of the Bt toxin against the pests that are prevalent. In the case of cotton, for example, Bt controls three major pests, the tobacco budworm, cotton bollworm, and pink bollworm, and in areas where these are prevalent, such as Alabama, the take-up of Bt varieties in some years has been as high as 77% and farmers report applying much less insecticide as a result.

The other *Cry* gene that has been used in plant biotechnology is the *CryIIIA* gene of *B. thuringiensis* var. *tenebrionsis*. The CryIIIA protein is effective against beetles, and potato varieties containing the *CryIIIA* gene are resistant to infestation by the Colorado beetle. One such variety, NewLeaf, produced by Monsanto, was on the market in the United States of America for several years in the 1990s, but was withdrawn due to poor sales. The United States potato plantations are attacked by a number of pests that are not controlled by Bt, in addition to the Colorado beetle, and farmers turned to new, broad-range insecticides instead of the GM option. The NewLeaf variety also failed to find favor with fast-food chains, a key market for potatoes in North America.

In the United States of America, the responsibility for monitoring and controlling the use of most GM crops lies with the Animal and Plant Health Information Service (APHIS) within the U.S. Department of Agriculture. However, Bt crops are the responsibility of the Environmental Protection Agency, which argued that as it was already responsible for controlling the use of the conventional Bt pesticide, it had to have control of the use of Bt crops as well. The agency considered the emergence of resistance to Bt to be a significant risk and insisted that farmers using Bt crops would have to plant "refuges" of a non-GM variety so that insects that had developed resistance to the Bt toxin would not be at a selective advantage. Although not readily accepted by farmers when it was introduced, this policy appears to

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have worked well. However, Bt crops are now being grown in developing countries where monitoring and enforcement are likely to be more difficult than in the United States of America, and the risk of insects developing resistance to Bt remains a concern. As with GM-HT crops, an increase in the diversity of insect resistance systems would be highly desirable, but there is no sign of an alternative to Bt being launched in the near future.

Fruit shelf-life

Extending the shelf-life of fruit would be of obvious benefit to the whole fresh fruit supply chain, from grower to consumer. Various strategies have been developed to do this through GM, mainly focused on the production of or response to the plant hormone, ethylene, which induces the ripening process. The first GM variety of any kind to be marketed was the "Flavr Savr" tomato, which was developed by Calgene, subsequently acquired by Monsanto, and marketed in the United States in 1994. "Flavr Savr" had reduced activity of polygalaturonase (PG), one of the enzymes that breaks down pectin, as a result of antisense inhibition (Sheehy et al. 1988). Pectin is a complex group of polysaccharides based on galacturonic acid and rhamnose, with various sugar side-chains, and its breakdown is part of the fruit softening process. "Flavr Savr" was not a success and was soon withdrawn. However, a competing group led by Don Grierson at the University of Nottingham and Wolfgang Schuch at Zeneca (now Syngenta) had developed very similar technology (Smith et al. 1988). This was eventually commercialized in the form of tomato paste made from GM tomatoes in which PG activity was reduced by cosuppression. The GM tomatoes had a higher content of solids than conventional varieties, reducing waste and processing costs in paste production and giving a paste of thicker consistency. The product went on the market in many countries and even proved popular in the United Kingdom where over two million cans of it were sold between 1996 and 1999.

Reducing PG activity slows down the response to ethylene. An alternative strategy is to reduce the production of ethylene, resulting in the fruit developing to the point where it would normally start to ripen and no further, allowing the farmer to harvest it all at once. Ripening is then induced by spraying the fruit with ethylene. Tomato has again been the main target for the development of this technology and several methods have been shown to work. One is to suppress the gene that encodes the enzyme aminocyclopropane-1-carboxylic acid (ACC) synthase, one of the enzymes in the ethylene synthesis pathway (Hamilton et al. 1990). A tomato variety of this type was developed by a company called DNA Plant Technologies and marketed in the United States of America in the 1990s under the trade name "Endless Summer". However, the variety was withdrawn from sale because of disputes over patenting.

An alternative method with a similar outcome is to add a gene that encodes an enzyme called ACC deaminase. This enzyme interferes with ethylene production by breaking down ACC. Tomatoes of this type have been developed by Monsanto using a gene derived from a soil bacterium called *Pseudomonas chlororaphis* (Klee et al. 1991), but so far have not been marketed. A third method targets another of the precursors of ethylene, S-adenosyl methionine (SAM), by introducing a gene that encodes an enzyme called SAM hydrolase, which breaks down SAM (Good et al. 1994); this strategy was developed by Agritope, Inc., Portland, Oregon using a viral SAM hydrolase gene, but again has not been marketed.

Clearly, GM tomatoes with delayed ripening traits have had a chequered history in the west. The technology works, but tomatoes are a relatively minor crop and possibly do not generate the revenue required to sustain a GM program under current constraints of regulation and public acceptance. The technology could be applied to other fruit crops, but the same problems would probably arise. However, slow-ripening GM varieties of tomato and papaya (*Carica papaya*) are being grown commercially in China.

Disease resistance

Herbicide tolerance and insect resistance currently dominate the plant biotechnology market. However, some other traits have been used successfully in some crops and in some areas. Virus resistance is an example and this technology could have important applications in developing countries in the future, where viruses, such as *Cassava mosaic virus* and *Feathery mottle virus*, among others, are responsible for the deaths of millions of people every year through the destruction of vital food crops.

One way of engineering plants to be resistant to viruses is to exploit the phenomenon of cross protection, in which infection by a mild strain of a virus induces resistance to subsequent infection by a more virulent strain. Cross protection involves the coat protein of the virus and genetically modifying a plant to make a viral coat protein invokes a similar response. This technology has been used successfully to engineer papaya to be resistant to *Papaya ringspot virus* (PRSV) and a virus-resistant GM variety has been grown in Hawaii since 1998 (Gonsalves 1998; Ferreira et al. 2002). The cultivation of this variety remains controversial even now because some important markets were lost as a result, notably that of Japan, but

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some commentators claim that the GM variety saved the papaya industry.

Virus resistance can also be achieved by using gene suppression techniques to block the activity of viral genes when the virus infects. This technique was used by Monsanto in the 1990s to engineer resistance to *Potato leaf roll virus* (PLRV) into potato by blocking expression of the viral replicase gene (Lawson et al. 2001). A variety containing this trait and the Bt insect-resistance trait was marketed under the trade name NewLeaf Plus but, like the NewLeaf variety, this was not successful and was withdrawn. However, virus-resistant papaya, tomato, and sweet pepper (*Capsicum annuum*) are being grown commercially for cultivation in China, and Brazil has just approved for cultivation a GM common bean (*Phaseolus vulgaris*) (known as pinto bean in Brazil) that is resistant to *Bean golden mosaic virus* (Bonfim et al. 2007). The latter is an example of research in the public sector, in this case the Brazilian Agricultural Research Corporation (EMBRAPA), leading to the development of a crop grown in the main by poor farmers. Kenya has also field-tested a GM sweet potato (*Ipomoea batatas*) variety engineered to be resistant to *Feathery mottle virus* and a GM cassava (*Manihot esculenta*) variety that is resistant to *Cassava mosaic virus*, but so far these varieties have not been approved for cultivation.

Improved resistance to diseases caused by fungi and oomycetes is also a target for crop biotechnologists, and there have been several field trials in Europe of GM potato lines engineered to be resistant to the oomycete *Phytophthora infestans*. One of these lines was produced by BASF using a gene called *RB* from a wild potato species, *Solanum bulbocastanum* (Song et al. 2003). Despite suffering vandalism of field trials in Ireland and Europe, BASF had obtained sufficient data to apply for consent to market their blight-resistant potato for cultivation in Europe. However, in 2012, the company announced that it was abandoning attempts to develop GM crop varieties for Europe and would be concentrating on easier markets, so this technology is likely to be transferred to North American potato varieties. Two potentially blight-resistant GM potato lines have also been developed at the John Innes Centre in Norwich, U.K., one containing the *Rpi-vnt1.1* gene from *Solanum venturii*, (Foster et al. 2009), the other the *Rpi-moc1* gene from *Solanum mochiquense* (Smilde et al. 2005).

These GM potato lines have been engineered to express a gene from a wild potato species, and genetic modification is undoubtedly the safest and most efficient method for moving a gene from a wild potato to a cultivated potato breeding program. This is because wild potato species are diploid, while cultivated varieties are tetraploid, making the crossing of wild and cultivated potato species extremely difficult. Furthermore, potatoes contain glycoalkaloids such as solanine and chacocine, probably to deter insects and other herbivores and to defend against fungal infection. These extremely large organic compounds are toxic, causing nausea, dizziness, vomiting, diarrhea, heart arrhythmia, and in extreme cases, coma and death. Their levels in the tubers of cultivated potatoes have been reduced by breeders, but the tubers of wild potato species usually contain much higher levels and consequently are not considered fit for human consumption.

An alternative approach to the challenge of engineering fungal resistance into crop plants is to modify them with genes that express fungicidal proteins. Examples are genes encoding the enzymes chitinase and ß-glucanase, both of which attack the cell walls of fungal hyphae as they enter the plant. Transgenic plants containing genes for these enzymes have been reported to have increased resistance to pathogenic fungi under experimental conditions, (Punja and Raharjo 1996; Anand et al. 2003), although success of the strategy appears to vary from species to species.

Modified oil content

Plant oils contain a variety of fatty acids with different chain lengths and degrees of saturation (Table 1). Well-known plant fatty acids include lauric acid (12 carbon atoms, no double bonds; 12:0) and palmitic acid (16:0), which are found in coconut and palm kernel oil, and stearic acid (18:0), a major component of cocoa butter. Common unsaturated fatty acids include oleic acid (18:1), which contains a double bond at position 9 with respect to the methyl (omega) end of the molecule (*n-9*), and is the major constituent of olive and oilseed rape oil. Linoleic acid (LA) (18:2, *n-6*) (Fig. 3) is found in safflower (*Carthamus tinctorius*), sunflower, and maize oil, and makes up about 20% of oilseed rape oil. Gamma linolenic acid (GLA) (18:3, *n-6*) (Fig. 3) is another *omega-6* fatty acid and is identical to LA except that it has an additional double bond at *n-12*; starflower (borage) (*Borago officinalis*) oil contains more GLA than any other, but evening primrose (*Oenothera biennis*) oil is also a good source. Alpha linolenic acid (ALA) (18:3, *n-3*) is similar to GLA, but crucially, the double bonds are situated at different positions, with the first at *n-3* with respect to the methyl end, making ALA an omega-3 fatty acid.

LA is an essential fatty acid because humans do not synthesize it, and GLA is often described as essential because it can only be synthesized from LA; GLA may be particularly important for people with diabetes, who appear to convert LA to GLA inefficiently. LA and GLA are precursors for longer chain omega-6 fatty acids such as arachadonic acid (AA; 20:4, *n-6*) (Fig. 3) that are not present in plant oils. AA is present in the phospholipids of cell

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Table 1. Typical fatty acid content (%) of some plant oils (Halford 2012), where total does not equal 100%, balance is made up of minor constituents that are not shown.

	Caprylic acid, 8:0	Capric acid, 10:0	Lauric acid, 12:0	Myristic acid, 14:0	Palmitic acid, 16:0	Stearic acid, 18:0	Oleic acid, 18:1; Omega-9	Linoleic acid, 18:2; Omega-6	α-Linolenic acid, 8:3; Omega-3	γ-Linolenic acid, 8:3; Omega-6	Erucic acid, 22:1; Omega-9
Oilseed rape (edible)					4	2	61	21	11		
High lauric GM oilseed rape			40		3	1	40	15			
Oilseed rape (industrial)				1	3	1	14	11	11		54
Sunflower					9	7	10	74			
Palm				1	44	5	39				
Soybean					11	4	23	54	7		
High oleic acid GM soybean					11	4	80				
Coconut	8	7	48	18	9	3	6				
Linseed					6	4	20	16	54		
Evening primrose					6	2	8	70		10	
Starflower (borage)					10	4	8	37		23	
Maize					12	2	30	54	1		
Peanut						18	47	29			
Olive					14	2.5	69	12			

membranes and is abundant in the brain and muscles; it is also used to make eicosanoids, such as prostaglandins, leukotrienes, and isoprostanes, which have a variety of roles in the body. Dietary intake is from meat, eggs, and dairy products, so it is not present in the diet of vegans, for whom consumption of adequate amounts of LA or GLA from plant sources is particularly important.

Plant oils have long been used for industrial as well as food uses, and this has risen greatly in the last decade or so with the increased use of oils to make biodiesel, usually after esterificatrion with methanol to create fatty acid methyl esters (FAMEs). Erucic acid (22:0) is an example of a plant oil with many industrial applications, from transmission oils to health care products. It is toxic, and its presence in oilseed rape oil is one of the reasons why oilseed rape oil used to be regarded as unfit for human consumption. Plant breeding, including an intense program of mutagenesis, reduced the levels of erucic acid to the point where oilseed rape oil was considered edible, giving rise to an alternative name for the crop, canola (**Can**adian **o**il, **l**ow erucic **a**cid), which was adopted throughout the Americas. Edible varieties typically contain oleic acid (60%), LA (20%), and ALA (10%), with palmitic, stearic, and other fatty acids together accounting for the other 10%.

Genetic modification has been used to change oilseed rape oil further: Calgene developed a GM oilseed rape variety containing a gene from the Californian Bay plant (*Umbellularia californica*) that encodes an enzyme that causes premature chain termination of growing fatty acid chains, resulting in the accumulation of high levels (40%) of lauric acid (12:0) (Voelker et al. 1992). This variety was introduced in 1995 to compete in the detergents and shampoo market, but failed to gain ground against palm and coconut oil. The technology is now owned by Monsanto, but there is no cultivation of high lauric acid oilseed rape at present.

More success has been had in the genetic modification of soybean to alter its oil content. A GM variety, Plenish, in which the activity of a gene encoding a delta-12 desaturase enzyme that converts oleic acid to LA is reduced, has been produced by PBI, a subsidiary of DuPont. This variety accumulates oleic acid to approximately 80% of its total oil content, compared with 20% in non-GM varieties (Kinney 1997). Monsanto also has a high oleic acid variety, Vistive, on the market; this is a GM variety because it carries the glyphosate tolerance trait, but the high oleic acid trait was developed by mutagenesis, not GM. The high oleic acid oil from these varieties is claimed to be much more stable during frying and cooking, less prone to oxidation and therefore less likely to form compounds that affect flavor (Mounts et al. 1994). Normally, soybean oil is hydrogenated to prevent

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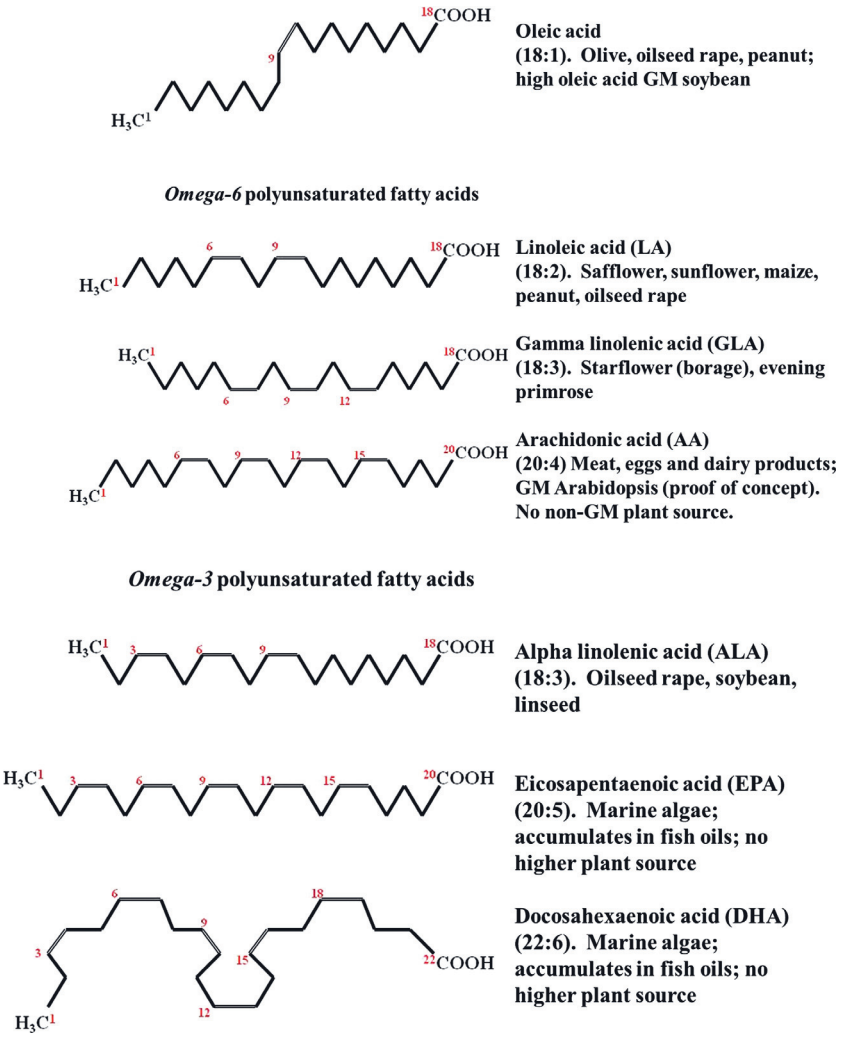

Figure 3. Diagram illustrating the structures of some well-known fatty acids. Oleic acid is an 18-carbon mono-unsaturated fatty acid found in olive and other naturally occurring oils and in GM soybean varieties with modified oil content. Linoleic acid and gamma linolenic acid are 18-carbon omega-6 poly-unsaturated fatty acids present in some plant oils, while arachidonic acid is a 20-carbon omega-6 fatty acid that is not present in any plant oils. The 18-carbon omega-3 fatty acid, alpha linolenic acid, is also shown, along with the longer chain omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, that are synthesized by marine algae and found in fish oils. The numbers in red indicate the first and last carbon in the chain, taking the carbon at the methyl end of the chain as 1, and the positions of double (unsaturated) bonds between the carbon atoms.

oxidation, but this can create trans fatty acids, which contain double bonds in a different orientation to the cis fatty acids present in natural plant oils. Trans fatty acids raise blood cholesterol, potentially contributing to cardiovascular disease, and U.S. law now requires that trans fatty acid content be included in the information in food labels. The better heat stability of high oleic acid soybean oil also makes it suitable for industrial uses (Cahoon 2003).

Farmers only gain from growing varieties like these if they get a premium price for them. The introduction of labeling legislation for trans fatty acids in 2005 led to a significant increase in adoption of high oleic varieties, but it has undoubtedly been more difficult to sell "output" as opposed to "input" traits to farmers and the area of cultivation of high oleic varieties remains relatively small. One potential problem with these varieties is that, while reducing trans fatty acid content of foods is a worthy target, LA and ALA are important dietary fatty acids, and it is unlikely that consumers will be aware and understand the significance of the differences between conventional soybean oil and oil from these GM varieties.

Genetic modification is also being used to enhance the levels of long chain polyunsaturated fatty acids (LC-PUFAs) in crop plant oils or to produce valuable LC-PUFAs that are not normally present in plant oils. GLA was an early target in this work because the plant species for which it is a major oil constituent, namely evening primrose and starflower (borage), make poor crops. In a proof of concept experiment, tobacco has been engineered to produce GLA using a gene from starflower encoding a delta-5 desaturase (Sayanova et al. 1997), and engineering of Arabidopsis with multiple transgenes has shown that it is possible to produce arachidonic acid itself in plants (Qi et al. 2004). However, the technology has not yet been commercialized.

Omega-3 LC-PUFAs such as eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6) (Fig. 3) are also targets for biotechnologists. The only

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dietary source for omega-3 LC-PUFAs at the moment is marine fish oil. The human body can synthesize them from ALA, but the efficiency is low: approximately 5% in men and slightly higher in women. Eicosanoids derived from omega-3 LC-PUFAs act to modulate platelet aggregation and immuno-reactivity. There is also increasing evidence of the effectiveness of omega-3 LC-PUFAs in the prevention of cardiovascular disease, metabolic syndrome and type 2 diabetes. Furthermore, fetal development is now known to require omega-3 LC-PUFAs and both EPA and DHA are commonly added to infant formula milk.

EPA and DHA are in fact made by marine algae, not fish; they are present in fish oils only because they accumulate through the marine food chain. Marine fish stocks are in decline and farmed fish, like marine fish, acquire EPA and DHA in their diet, through being fed marine fish meal, so farmed fish currently cannot be considered to be a sustainable source of these fatty acids either. Finding a sustainable alternative source is therefore essential and the development of GM plants producing EPA and DHA is an obvious solution. Proof of concept has been demonstrated in Arabidopsis using multiple transgenes (Qi et al. 2004) and several biotech companies claim to be close to launching commercial varieties of soybean and oilseed rape that produce EPA and DHA.

Modified Starch, Biofuels, and High Lysine Animal Feed

Nonfood uses of starch include the manufacturing of paper, adhesives, gypsum wall boards, and textile yarns, among many others. Starch is, of course, a glucan, made up of chains of glucose units, but it comprises two components, amylose, consisting of long, unbranched chains of glucose units, and amylopectin, consisting of branched chains. A difficulty in using starch for industrial purposes is that amylose and amylopectin have different characteristics and have to be separated or modified chemically before use. Amylose, for example, has gelling properties that are undesirable in some processes. The production of GM potatoes in which the starch was composed almost entirely of amylopectin as a result of reduced activity of a granule-bound starch synthase was reported in 1991 (Visser et al. 1991) and BASF used this technology to produce a commercial GM potato variety, marketed as "Amflora".

Amflora was developed for the European market and was mired in the EU's regulatory processes covering the use of GM crops for over a decade. It was finally approved for cultivation in 2010 and BASF cultivated Amflora in Germany and Sweden in 2011 to produce seed potatoes. However, BASF announced in 2012 that it was withdrawing from plant biotechnology in Europe altogether and concentrating on markets elsewhere. Currently, it has no plans to continue with Amflora.

Starch can be used to produce sugars through enzymatic digestion, and maize starch, for example, has been an important source of sugars in the food industry in the United States of America for many years. More recently, there has been a huge increase in the use of sugars derived from starch for the production of ethanol for transport fuel. In the United States of America, for example, bioethanol production from maize starch saw an annual growth rate of 25% between 2003 and 2007 and in 2010 bioethanol production took a third of the U.S. maize crop. Several plants designed to produce biofuel from wheat grain have been or are being built in the United Kingdom, the largest expecting to take over a million tonnes of grain per year and produce up to 400 million liters of ethanol and 350 thousand tonnes of animal feed coproduct. One fifth of the U.K.'s wheat harvest could be used for fuel production by 2015. However, development of these plants is currently stalled in the face of cheap imported ethanol and it is not clear how the industry is going to develop.

Bioethanol production from cereal grains generally uses a "dry-grind" process in which the entire kernel is ground into a coarse flour, then slurried with water. The resulting mash is then cooked, treated with enzymes, fermented, and distilled. The first enzyme to be added is α-amylase to produce maltotriose, maltose, and limit dextrin (a mixture of branched and unbranched glucans) in a process known as gelatinization and liquefaction. Gluco-amylases are then added to achieve saccharification, in which smaller sugars are produced, ready for fermentation. The yield of sugar from starch is the most important cost determinant in bioethanol production and is a target for biotechnologists. Syngenta have already produced a GM maize variety that it claims gives a better yield of ethanol in the dry-grind process (Johnson et al. 2006). It contains a gene, *amy797E*, from a thermophilic bacterium, *Thermococcales spp.*, that encodes a highly thermostable α-amylase. It was deregulated by the U.S. authorities in early 2011.

Another target for improvement of starchy crops used for bioethanol is the quality of the high-protein coproduct used in animal feed. Cereal grain usually has to be supplemented in animal feed because it contains insufficient amounts of the essential amino acid lysine. Renessen, a joint venture between Cargill and Monsanto, has produced a maize variety with high lysine levels through expression of a gene from a bacterium *Corynebacterium glutamicum* encoding a lysine-insensitive dihydrodipicolinate synthase (DHDPS) (Huang et al. 2005). Feedback inhibition of DHDPS is the major regulatory control for flux through the lysine biosynthesis pathway in plants, but the bacterial enzyme is not affected by lysine, allowing

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the amino acid to accumulate to high levels. Mavera is currently being grown entirely for U.S. domestic bioethanol/animal feed production. It also contains a triple stack of input traits (resistance to corn rootworm and the European corn borer and tolerance of glyphosate) and is an indication of where maize biotechnology in the United States is heading. As such, it should sound alarm bells for European maize growers and regulators.

Golden Rice

Vitamin A deficiency is common in children in developing countries who rely on rice as a staple food. It causes symptoms ranging from night blindness to those of xerophthalmia and keratomalacia, where the cornea and conjunctiva become extremely dry, wrinkled, thick and ulcerated, sometimes leading to total blindness. Over five million children develop these conditions annually and approximately 250,000 of them become blind. Vitamin A deficiency also exacerbates major killers of children, such as diarrhea, respiratory diseases, and measles, and improving Vitamin A status in children reduces death rates by 30–50%. However, the World Health Organisation's efforts to eradicate vitamin A deficiency have been hampered by the difficulty of reaching those in need.

One potential solution would be to distribute rice varieties that contain vitamin A or its precursor, β-carotene. There is no way of producing such a variety by conventional breeding, but a GM line containing β-carotene was developed over a decade ago by Ingo Potrykus, a biotechnologist at the Swiss Federal Institute of Technology in Zurich (Ye et al. 2000; Beyer et al. 2002; Potrykus 2003). Rice endosperm contains geranylgeranyl diphosphate, which is converted into β-carotene by three enzymes produced from different transgenes: phytoene synthase (*psy*) and lycopene β-cyclase genes from daffodil (*Narcissus pseudonarcissus*), and a phytoene desaturase (*crtI*) gene from the bacterium *Erwinia uredovora* (Fig. 4). The GM rice producing β-carotene was crossed with another line engineered with multiple genes to improve iron availability, including a phytase-encoding gene from *Aspergillus fumigatus* (Lucca et al. 2001). The high β-carotene/high available iron hybrid was called Golden Rice.

In a press release of February 2001, the pressure group Greenpeace described Golden Rice as "fool's gold", claiming that an adult would have to eat at least 3.7 kg of dry rice (12 times the normal intake of 300 g) to get the daily recommended amount of β-carotene. Potrykus responded that nutritional experts involved in the project believed that the levels of β-carotene present in Golden Rice would have a significant effect in preventing blindness and other symptoms associated with severe vitamin A deficiency. Greenpeace dropped the issue when the development of Golden Rice 2 was announced; this line had been produced by Syngenta in collaboration with Potrykus by replacing the daffodil phytoene synthase gene with one from maize and it contained many times more pro-vitamin A than Golden Rice 1.

The production of high β-carotene rice was first reported in 2000 (Ye et al. 2000), 12 years ago, and by 2001, Golden Rice was already being crossed into local varieties by centers such as The Rice Research Institute in Manila, The Philippines. However, the release of Golden Rice-derived varieties to farmers has been held up by governments concerned about their export markets in Europe and Japan, where resistance to GM crops has been strongest. In this case, therefore, European attitudes and over-regulation are costing thousands of lives in developing countries.

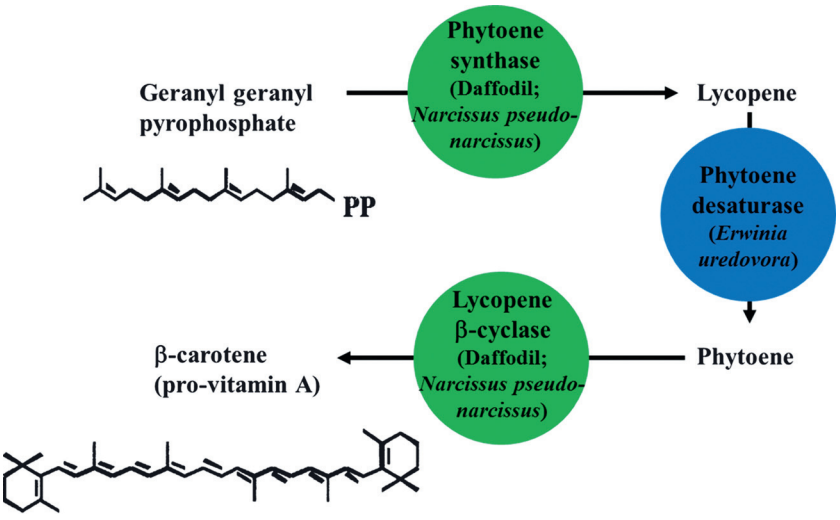

Figure 4. Diagram showing the biosynthetic pathway for β-carotene that was engineered into Golden Rice, from geranyl geranyl pyrophosphate, a precursor that is naturally present in rice endosperm (Ye et al. 2000). In Golden Rice 2, the phytoene synthase gene from daffodil is replaced with one from maize.

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Drought Tolerance

Plants may avoid late summer drought (typical of the U.K. and northern Europe) by growing, flowering, and setting seed before this time. They can also avoid becoming water-stressed by developing deeper and more extensive root systems. Plants have also evolved tolerance traits that enable them to survive even if they do become short of water. These response strategies differ from species to species and even between different varieties, developmental stages, organs, and tissue types, but most involve abscisic acid (ABA), which initiates a network of signaling pathways in response to water stress.

ABA signaling involves SNF1-related protein kinase-2 (SnRK2) and protein phosphatases of the PP2C family (Cutler et al. 2010) (Fig. 5); in the absence of ABA, PP2C inactivates SnRK2 by dephosphorylation of one of the serine residues in the activation loop (Umezawa et al. 2009). If ABA is present, the PYR/PYL/RCAR ABA receptors (Nishimura et al. 2010) bind to and inhibit PP2Cs, allowing the accumulation of active SnRK2s and subsequent phosphorylation of ABA-responsive element binding proteins (AREBPs) (Cutler et al. 2010). AREBPs (also known as ABFs) are a family of basic leucine zipper (bZIP) transcription factors that recognize the G-box binding sites known as ABA response elements (ABREs) present in some ABA-regulated genes (Cutler et al. 2010). Recent evidence suggests that ABA may also cause degradation of SnRK1, a protein kinase related to SnRK2 that is a master regulator of carbon metabolism (Coello et al. 2012), and calcium-dependent protein kinases have also been implicated in drought responses (Saijo et al. 2000).

Other transcription factors known to be involved in drought stress responses include dehydration-responsive element binding protein (DREB)-1 and -2, members of the zinc-finger homeodomain (ZFHD)-1, myeloblastosis (MYB), and myelocytomatosis (MYC) families, and the NAC family, which comprises no apical meristem (NAM), ATAF1 and 2, and cup-shaped cotyledon (CUC) transcription factors (reviewed by Semenov and Halford 2009). The action of AREBPs, DREB1, MYC, and MYB requires ABA, while that of DREB2, ZFHD1, and NAC does not. Over-expression of another transcription factor, plant nuclear factor-Y (NF-Y), has been shown to confer increased drought tolerance in maize in the field (Nelson et al. 2007). One of the areas of plant metabolism that is affected by these signaling pathways is carbohydrate metabolism, which plants manipulate to mitigate the effects of osmotic stress brought about by drought, for example, by interconverting insoluble starch or fructan with soluble sugars (reviewed by Halford et al. 2011).

Clearly, the genes involved in drought stress responses are potential candidates for manipulation to improve drought tolerance in crop plants. However, Monsanto, not for the first time, has taken the approach of using a bacterial gene in crop biotechnology and has developed drought-tolerant maize varieties Genuity VT Triple Pro and Genuity VT Double Pro, in collaboration with BASF, that express a *Bacillus subtilis* RNA chaperone, cspB (Castiglioni et al. 2008). These varieties look set to be made available to farmers in 2012.

Biopharming

Biopharming is the term applied to the use of GM plants to produce pharmaceuticals, vaccines, antibodies, and enzymes. It is one of the most exciting areas of plant biotechnology and there are already examples of projects that have been commercialized or are in the later stages of development. In 2007, for example, a Canadian company, Sembiosys, announced that it had modified safflower to produce insulin in its seeds. The possibility of producing vaccines and antibodies in GM plants has also been causing some excitement (Nicholson et al. 2006). Progress has been slow, but the company, ProdiGene, based in Texas, has produced an edible vaccine for transmissible gastroenteritis virus (TGEV) in pigs (Lamphear et al. 2004), while a monoclonal antibody, Guys 13, has been produced in tobacco (Ma et al. 1994). This antibody binds to the surface protein of *Streptococcus mutans*, the bacterium that causes tooth decay. The technology is now licensed to Planet Biotechnology Inc., Hayward, California and is undergoing clinical trials under the product name CaroRx™.

An example of the production of an enzyme for industrial uses is trypsin, an animal protease that has a variety of applications in research and the food industry. ProdiGene has engineered maize to produce bovine trypsin and is marketing the enzyme under the trade name TrypZean (Woodward et al. 2003).

Maize is, of course, an outbreeder, and it will also produce volunteers in subsequent crops if not prevented from doing so; its use in the production of pharmaceuticals and other products that should not enter the food chain is therefore something of a risk. In 2001, ProdiGene contracted a Nebraska farmer to grow an experimental GM maize variety and the next year, the farmer was allowed to plant soybeans destined for human consumption on the same land. A tiny amount (65 g) of GM maize material was discovered in the harvested soybean seed and the Food and Drug Administration ordered the soybean crop, worth $2.7 million, to be destroyed. No doubt ProdiGene and the U.S. regulatory authorities learned from this mistake, but the episode does highlight the issue of segregating crops producing pharmaceuticals from food crops.

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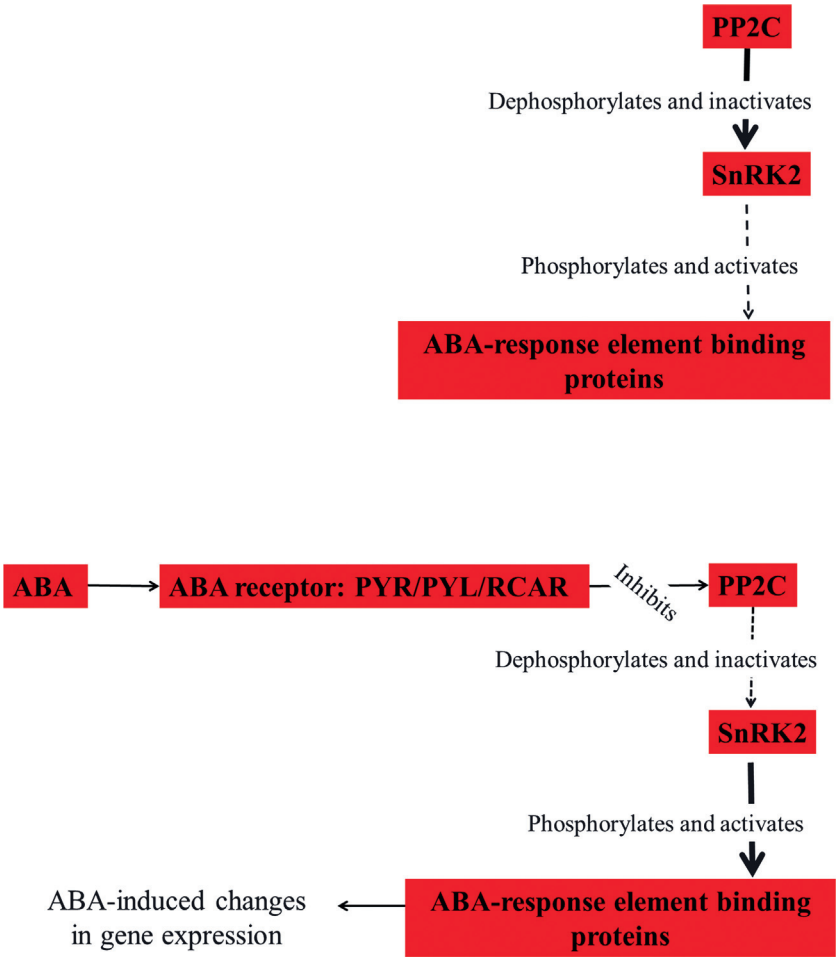

Figure 5. Diagram illustrating the abscisic acid (ABA) signaling pathway (Cutler et al. 2010), which is a target for biotechnologists in the improvement of crop stress tolerance. Top: Under normal conditions, the protein kinase SnRK2 is kept in an inactive state by the action of protein phosphatase PP2C. It is therefore unable to carry out its function of phosphorylating and activating ABA response element binding proteins (AREBPs). Bottom: When ABA is present, it is sensed by the PYR/PYL/RCAR receptor, which binds to and inhibits PP2C. Phosphorylated, active SnRK2 then accumulates and activates AREBPs, resulting in the expression of multiple ABA-responsive genes.

Current Status and Prospects

A striking aspect of the list of traits in the previous section is that it remains relatively short. As we approach the end of the second decade of GM crops, the number of traits being used successfully in commercial agriculture remains in single figures. There are others that may be on the way, such as salt and heat tolerance, and hypoallergenicity, but compared with the many thousands of GM lines that have been produced in plant genetic research, the number that have been developed into commercial varieties is tiny.

Nevertheless, some of the GM crops that have made it onto the market have been staggeringly successful. Data on the global use of GM crops have been compiled for several years by Clive James of the International Service for the Acquisition of Agri-Biotech Applications (ISAAA; www.isaaa.org). It is almost impossible to check the ISAAA's data for some countries, but for countries where independent data are available from other sources, the U.S.A., for example, the data sets are consistent. According to the ISAAA, the worldwide area of land planted with GM crops in 2011 was 160 million hectares (James 2011). Most (75%) of the global soybean production was GM, while 82% of cotton, 32% of maize, and 26% of oilseed rape production was GM. Other crops with some GM varieties being grown commercially included papaya, squash, tomato, alfalfa, tobacco, sweet pepper, poplar, potato, and sugar beet. GM crops were grown in 29 countries (Table 2), with the U.S.A., Brazil, Argentina, India, and Canada each planting more than 10 million hectares of GM crops, and China, Paraguay, Pakistan, South Africa, and Uruguay all planting more than a million hectares. Herbicide tolerance and insect resistance were the dominant traits, with approximately 95 million hectares of GM-HT crops, 43 million hectares of Bt crops, and 25 million hectares of crops in which the two traits were stacked. Second-generation (quality) traits are beginning to emerge, but these are being marketed and will almost certainly continue to be marketed on the back of successful, first-generation (input) traits.

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There are some notable absentees from the list of crop species for which a biotech market has been established. There is currently no GM wheat being grown commercially on a significant scale, for example, and while China has approved some GM rice varieties for cultivation, they have not yet been released to farmers. The only GM potatoes being grown in 2011 were of the Amflora variety, which has now been withdrawn. There are also some notable absentees from the list of countries where GM crops are being grown, including Japan and most countries in Africa (where arguably crop improvement is most needed) and Europe. The only significant cultivation of GM crops in Europe is that of GM maize in Spain, and even that amounts to less than 100,000 hectares. Glyphosate-tolerant soybean was grown in Romania up to 2006, when there were approximately 120,000 hectares of it, but this disappeared entirely in 2007 because Romania joined the EU.

There have been many studies on the economic impact of GM crops and I will not review them here. A report submitted to the EU by Kaphengst et al. (2011) of the University of Reading, the Swiss Federal Institute of Technology, Zurich and the Ecologic Institute of Berlin, which reviewed much of the available data, found that "*Positive economic effects of GM crops have been indicated in this study for several countries, which is in line with other review studies and explains the high adoption rates of GM crops in these countries. But the study also underlines……that such outcomes cannot be generalized across the globe*." Economic benefits are not the only reasons why farmers might choose to adopt GM crops, of course. Peace of mind certainly seems to have been a factor in the adoption of glyphosate-tolerant crops, for example; anecdotally, farmers appear to have enjoyed the security of knowing that they can deal with late season weed problems if necessary.

The situation in Europe remains extremely difficult, and the wet blanket of European attitudes and over-regulation extends well beyond Europe's boundaries because of the value of the huge European market. Only three African countries, for example, South Africa, Burkina Faso, and Egypt, have approved the use of GM crops, partly because of fears over losing access to the lucrative European market. The development of crop biotechnology in Europe itself has been stymied by several connected factors, first of which is the issue of regulation. Under the EU's directive, GM Food and Feed Regulation (EC) No. 1829/2003, the regulation of GM crop use and release is under the control of the European Commission. The EU recognizes two different types of field release of GM crops, one for research purposes only (a Part B release) and the other for commercial release (a Part C release). Consent for a Part C release may be granted for cultivation, food and feed use, or for food and feed use alone. While permission for a Part B release can be granted by an individual Member State, applications for a Part C release anywhere in the EU have to be approved by the European Commission.

Table 2. Global GM crop cultivation 2011 (James 2011 and pers. comm.).

Country	GM crop area	Crops
U.S.A.	69,000,000	Maize, soybean, cotton, oilseed rape, sugar beet, alfalfa, papaya, squash
Brazil	30,300,000	Soybean, maize, cotton
Argentina	23,700,000	Soybean, maize, cotton
India	10,600,000	Cotton
Canada	10,400,000	Oilseed rape, maize, soybean, sugar beet
China	3,900,000	Cotton, papaya, poplar, tomato, sweet pepper, oilseed rape, maize, soybean
Paraguay	2,800,000	Soybean
Pakistan	2,600,000	Cotton
South Africa	2,300,000	Maize, soybean, cotton
Uruguay	1,300,000	Soybean, maize
Bolivia	900,000	Soybean
Philippines	600,000	Maize
Australia	700,000	Cotton, oilseed rape
Myanmar (Burma)	300,000	Cotton
Burkina Faso	300,000	Cotton
Mexico	200,000	Cotton, soybean
Spain	100,000	Maize
Chile	<50,000	Maize, soybean, oilseed rape
Colombia	<50,000	Cotton
Costa Rica	<50,000	Cotton, soybean
Czech Republic	<50,000	Maize
Egypt	<50,000	Maize
Germany	<50,000	Potato
Honduras	<50,000	Maize
Poland	<50,000	Maize
Portugal	<50,000	Maize
Romania	<50,000	Maize
Slovakia	<50,000	Maize
Sweden	<50,000	Potato
Total	160,000,000	Alfalfa, cotton, maize, oilseed rape, papaya, poplar, soybean, squash, sugar beet, sweet pepper, tomato

Applications for Part C consent are assessed by EFSA. If EFSA approves the application, it is voted on by a working group with representatives from all 25 Member States, who may take advice from their own national experts. The U.K. representative, for example, may consult one or more of three advisory committees, the Advisory Committee for Releases to the Environment (ACRE), the Advisory Committee on Novel Foods and Processes (ACNFP), and the Advisory Committee on Animal Feedingstuffs (ACAF).

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Applications are voted on through a complicated system of Qualified Majority Voting (QMV), meaning that it is relatively easy for a minority of countries to block an application, and the European Commission failed to approve a single application between 1998 and 2004 because six member states, France, Italy, Denmark, Greece, Austria, and Luxembourg, blocked every one.

Even when the impasse was broken in May 2004 with the approval of an insect-resistant and gluphosinate-tolerant sweetcorn from Syngenta, it was only for food and feed use, not for cultivation, and Austria, France, Germany, Luxembourg, and Greece retained their own national bans in defiance of the European Commission. In 2006, the World Trade Organisation (WTO) ruled that the EU's position was illegal and also criticized the bans imposed by individual Member States. Nevertheless, Austria and Hungary still ban GM crops and their products outright, and there are still only two GM varieties approved for cultivation in Europe, the Amflora potato (now withdrawn) and Mon 810, the Bt maize variety grown in Spain and to a lesser extent in Romania, Slovakia, the Czech Republic, and Portugal. Efforts to develop new varieties for cultivation in Europe have all but been abandoned by biotech companies. Instead, companies are focusing on obtaining permission for GM crop products to be imported for food and feed use so that farmers elsewhere in the world can be reassured that the European market is open to their products.

Even small-scale field trials of GM lines are extremely difficult to run in Europe, and in the United Kingdom, for example, there have only been three in the last decade, an astonishing fact when set against the tens of thousands of field trials that have been carried out in the United States alone during the same period. The approval process is long and expensive, and field trials attract national and local headlines and a hostile reaction from activists. Figure 6 shows the drilling of a field trial at Rothamsted Research in the United Kingdom to assess aphid, predator, and parasitoid behavior in wheat producing an aphid alarm pheromone (Beale et al. 2006). The trial is protected by fencing and a 24-h security presence to prevent vandalism, which may seem bizarre, given that 160 million hectares of GM crops are being grown elsewhere in the world, but is necessary if the trial is to survive.

There is no scientific justification for the continuation of Europe's restrictive regulatory framework. There is a broad scientific consensus that GM is not inherently more risky than other methods in plant breeding; indeed, it is arguably more predictable than techniques such as random mutagenesis. A comparison of the metabolite profiles of three GM wheat lines and their non-GM parents grown at different sites in the United Kingdom over 3 years, for example, found that the differences in grain composition caused by site and year of cultivation were greater than those caused by the genetic modification (Baker et al. 2006). Another study compared global gene expression patterns in transgenic and conventionally bred wheat lines and found that the differences between conventionally bred genotypes were much larger than those between GM and non-GM genotypes (Shewry et al. 2007).

Even if a GM crop or crop product makes it onto the market in Europe, it must comply with labeling controls covering GM foods and feed. These were introduced in Europe in 1997 and extended in 2004 through directives on the regulation of GM food and feed (1829/2003) and the traceability and labeling of GMOs (1830/2003). These regulations require that any food containing material from GM crops must be labeled, unless the GM material is present through accidental mixing and does not exceed 0.9% of the total. This zero tolerance rule for products that have been approved elsewhere but are not yet approved in Europe (the issue of asynchronous approvals) is becoming increasingly unworkable as the amount of GM material in imported food and feed increases. The regulations cover animal feed, but not meat, dairy or other products from GM-fed animals or enzymes produced in GM micro-organisms and used widely in the production of cheeses, yogurts, and other foods.

Biotech companies also have to consider whether or not their product will find a market. In the United Kingdom, consumer hostility to GM crop products has declined; indeed, the proportion of respondents to a Food Standards Agency survey who, unprompted, listed GM food as a concern fell from 18% in 2004 to only 4% in 2011 (Food Standards Agency 2012). Nevertheless, the retail and food sectors in the United Kingdom and the

Figure 6. Field trial of GM wheat at Rothamsted Research in the United Kingdom in 2012. The small trial is one of a handful that has been attempted in the United Kingdom in the last decade and is surrounded by security fencing to prevent vandalism. Picture kindly provided by Rothamsted Research.

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rest of Europe remain extremely reluctant to use GM products, fearful of consumer fears being whipped up again by pressure groups who remain implacably opposed to GM. The animal feed market, on the other hand, has been using GM products on a large scale for the best part of a decade, because it has become difficult and expensive to source non-GM soybean, maize, and other essential raw materials.

This situation has arisen because science lost the GM debate. The consequences have been severe: Currently no GM crops are being developed specifically for European conditions or the European market; the biotech industry is focused on obtaining consent for import of more GM crop products into Europe, not for GM crop cultivation; European farmers are increasingly disadvantaged in a competitive global market, competing with GM crops, but unable to use them; biotech multinationals have largely moved out of Europe and the home-grown industry has been lost; the U.S., China, India, Brazil and others have a huge lead over Europe in a key 21st-century technology.

At some point, the debate will have to be reopened and the hearts and minds of the European public won over. The context and urgency of the debate are very different from what they were in the late 1990s: World population passed seven billion in 2011; increased prosperity and the ability to pay for a better diet in China, India and elsewhere are pushing up global food demand; severe weather events such as the droughts in Australia in 2006–2007 and Russia in 2010 may be the portent of things to come as the climate changes; biofuel is competing with food for crop products; peak oil (the point when the maximum rate of global petroleum extraction is reached, after which the rate of production enters terminal decline) is approaching and current crop yields are heavily fossil fuel-dependent; and there are concerns about fresh water supply, soil erosion, salination, pollution, and the loss of agricultural land to other uses. All this has led to an upward trend in food prices. In December 2010, the London International Financial Futures and Options Exchange (LIFFE) wheat price hit a record of £200 per tonne. It has fallen back since, but is currently still at a historically high price of £178 for May 2012. The era of cheap food and global food surpluses has already ended, and if we are to have food security in the coming decades, plant breeding will have to play its part and plant breeders will have to be able to use every tool that is available, including biotechnology.

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