Genetically modified (GM) plants have new properties such as herbicide tolerance, protective mechanisms against insects, and the ability to synthesize new compounds. Knowledge of metabolic pathways and the chemistry behind them is crucial for the successful production of GM plants with improved characteristics. Let us take a look at how it all began, how such plants are produced and detected in foods.

Products derived from genetically modified microorganisms (e.g., bacteria, yeasts) have been used for the production of such commodities as yogurt, cheese, and certain vaccines since 1982. In the USA, GM plants were approved for food use as early as 1992 and major staple crops (maize, rape seed, and soybeans) followed shortly. In 1997, GM soybeans (Monsanto Roundup Ready) already represented approximately 10% of the total soy harvest in the USA and in 1999 it was 54% (1). Soybeans are one of the most versatile staple crops, used as an ingredient in more than 25,000 foods (2). GM maize resistant to corn borer (Novartis Maize Bt 176, MON 810 Bt 11, among others) is also used in a wide range of foods.

Transformation of plants is done by inserting DNA into a single plant cell, which is then regrown into a complete plant. There are two procedures that are mainly used to transform these plants. One is the "gene-gun" method depicted in Figure 1; the other, the so-called "transfection" with an agrobacterium that infects the plant and thereby transfers its DNA to the plant. But the introduction of just the gene that codes for the new property is not enough. In order for the gene to be expressed as a protein, other elements must also be included in the GM construct (Fig. 2): a promoter, or start signal; the gene itself; and a terminator, or stop signal. Many times a marker gene is also included to allow selection of transformed cells from nontransformed cells during the transformation process.

Why Have GM Crops Been Created?

The first generation of GM crops was created for two main reasons: to increase crop yield and to improve the food quality. The yield of a crop can be increased by introducing resistance to herbicides, insect pests, and certain diseases, by enhancing growth, or by improving storage, transport, and harvest characteristics. Food quality can be improved by increasing the synthesis of desired substances and/or decreasing the synthesis of undesired compounds such as antinutrients. In addition, by alteration of metabolic pathways, new substances can be synthesized. As an example, genetically modified oilseed rape has been created with a high oleic acid content, which is thought to be beneficial in the human diet.

Of course, the third reason for creating genetically modified crops must not be forgotten: the economic advantage to the biotechnology industry, the farmer, and potentially, the consumer.
Three of the best-known GM traits are described below.

The Monsanto Roundup Ready soybean line 40-3-2 has a tolerance to the herbicide glyphosate, the active ingredient in Roundup herbicide. This is accomplished by expressing the gene for an enzyme (5-enolpyruvylshikimate-3-phosphate synthase or CP4-EPSPS, derived from Agrobacterium sp) in the plant. Glyphosate controls weeds via the inhibition of the enzyme EPSPS. Glyphosate-tolerant soybean (GTS) line 40-3-2 soybeans express a CP4-EPSPS enzyme tolerant to the glyphosate, thereby conferring tolerance to Roundup. EPSPS is involved in the shikimic acid pathway for synthesis of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) in plants and microorganisms and is therefore ordinarily present in food derived from plant sources. It catalyzes the formation of 5-enolpyruvylshikimate-3-phosphate, an intermediate in the aromatic amino acid pathway, from shikimate-3-phosphate and phosphoenolpyruvate. Inhibition of EPSPS by glyphosate directly arrests aromatic amino acid synthesis, resulting in the reduction of protein synthesis and inhibition of growth. Glyphosate tolerance can be conferred on plants and microbes by either overproduction of EPSPS or the use of glyphosate-tolerant EPSPS. Genes from numerous EPSPSs have been cloned, and active-site domains are conserved among them. EPSPSs from a number of bacteria exhibit tolerance to glyphosate.

Novartis Bt 176 maize (corn), known under the trade names “Maximizer” and “Knockout”, was created by introduction of a gene from Bacillus thuringiensis (Bt). The gene encodes the Bt toxin, Cry1Ab, a protein active against insects of the order Lepidoptera (which includes the corn borer), whose cells have receptors that “recognize” it. It is ingested when insects feed on the plant. The protein is not toxic to species whose cells do not have receptors for it and therefore cannot interact with it.

Laurate canola lines were created by introducing the bay thioesterase (TE) gene isolated from California bay, Umbellularia californica, into the oilseed plant Brassica napus. The introduced gene is expressed only in the seeds of B. napus, which contain a seed-specific promoter, mann. TE encodes the 12.0 thioesterase that results in the accumulation of laurate, the C12 saturated fatty acid, in canola seeds. Laurate canola offers the agricultural diversification much needed by American farmers in the Upper Midwest and Southeast. It may provide a domestic source of a raw material worth approximately $400 million for soaps and detergents. It is an alternative to imported coconut and palm oil and may indirectly benefit many other businesses.

Detection

Why is detection of GM crops necessary? Mainly, to allow consumers to make an informed choice (3) and to comply with labeling regulations.

Consumers Want to Make Informed Choices

Although legislation governs the release of novel foods and a strict approval process is in place that includes the safety assessment of every new GM plant, there is still widespread concern about plant-derived GM products. This was sharply increased by TV and press coverage of the topic as well as by actions of Greenpeace and Friends of the Earth, which raised awareness of the issue. The reasons why genetic engineering in foodstuffs is rejected are complex. They include ethical concerns (4), a “no” to biotechnology in agriculture, and fear of possible health (5) or environmental (6) risks of transgenic organisms.

Labeling Regulations

The central regulations for labeling genetically engineered food or food ingredients in the European Union are the European Commission (EC) Novel Food Regulations 258/97 and 1139/98 (7). These require labeling if “any characteristic or food property such as composition, nutritional value or nutritional effects or intended use of food…renders a novel food or food ingredient no longer equivalent to an existing food or food ingredient” or if DNA or protein of Monsanto Roundup Ready soya and Novartis Bt 176 maize can be detected. On 10 April 2000, two new regulations came into force: the 49/2000/EC, which introduces a 1% threshold for labeling of products derived from identity-preserved sources (e.g. organic, non-GM grain), and the 50/2000/EC, requiring labeling of flavors and additives (the latter having previously been exempt from labeling). In Switzerland, a 1% threshold for labeling of products containing GM material had been introduced in June 1999 and Norway had had a 2% threshold for several years.

A majority of members of Mexico’s parliament also voted for labeling of foods containing transgenic material. Japan announced the introduction of a 5% threshold for labeling in 2001. However, some lobby groups push for a lower threshold, similar to the one of the European Union.

Negative List

EC Regulations 1139/98 and 49/2000 also establish the principle of a negative list for “specific foodstuffs in which neither protein nor DNA resulting from genetic modification is present”: “A list of products not subject to the additional specific labeling requirements shall be drawn up under the procedure laid down in Article 17 of Directive 79/112/EEC, taking account of technical developments, the Scientific Committee on Food and any other relevant scientific advice” (8). At present, a number of food ingredients and final products such as modified starches and soy oil are being considered for possible inclusion on such a negative list (9).

Ways of Detecting GM Organisms

Several factors must be assessed when choosing a strategy for analysis: specificity and sensitivity, feasibility and practicality, potential for validation, and cost and time efficiency. For the detection itself, other parameters are crucial: the availability of validated methods, availability of reference material, the nature of the food matrix, the degree of degradation, and the homogeneity of the sample.

The food matrix contains a wide range of substances such as lipids, fatty acids, and polysaccharides in addition to DNA and protein. Some of these can negatively affect detection. The presence of certain plant polysaccharides can inhibit the polymerase chain reaction (PCR) (10, 11), producing a false negative result when this method is used unless appropriate controls are included. That is, although GM material is present, the PCR cannot take place because it is inhibited by the plant polysaccharides.

The degree of degradation of the test material is also important. If the protein or DNA to be detected is too degraded to be recognized by antibodies or primers, respectively, there...
is again potential for a false-negative result. This too can be prevented by applying appropriate controls.

Sample homogeneity is crucial because in most cases only a subsample of the product is taken. If the GM material is not uniformly distributed throughout the sample, there is again potential for a false-negative result.

The detection of genetically modified plants can have several targets: newly synthesized compounds such as fatty acids, a newly expressed protein, or nucleic acids (DNA or RNA). DNA is the blueprint of each cell that is transcribed into the less stable messenger RNA (mRNA), which is then translated into a protein.

During processing of a food containing GM material, one or several steps (e.g., heating, pressure) can induce degradation of the target compounds. Because RNA is a highly unstable molecule that usually does not survive processing, it is not considered a suitable target for detection. Moreover, in the future it will be possible to carry out genetic modification by introducing regulatory DNA sequences, which are not transcribed into mRNA.

For GM food samples in which genetically modified DNA is no longer present, two types of detection are possible. If a new chemical compound that does not occur naturally in the food (e.g. a novel fatty acid) is produced as a consequence of the genetic modification of metabolism, it may be tested for by chemical (GC–MS, NMR, FPLC, etc.) or immunological (ELISA, Western blot) analyses. Common problems with these methods are the extensive purification procedures required for GC–MS and NMR chemical analysis and cross-reactivity (one antibody reacting with multiple antigens) in the immunological reactions.

There are several options for testing for the gene product (protein).

Chemical detection of the transgenic protein using GC–MS, FPLC, or CE. In all cases, the expression level of the protein will limit the usefulness of these methods. In approved crops, the expression levels of transgene products in parts of plants used for human consumption are below 0.06%; most are in the lower parts per million range and some even in the parts per billion or parts per trillion range. However, FPLC has been used successfully for the detection of chymosin produced from GM organisms. Here, the chymosins from bacteria and cattle have different characteristic FPLC profiles, which can be used for identification (2).

Immunologic detection of the transgenic protein using Western blot or ELISA. For a Western blot, the protein is extracted from a food or food ingredient and immobilized on a nitrocellulose membrane. The proteins bound to the membrane are then immersed in a solution containing an antibody that specifically recognizes the target protein (e.g. the protein from B. thuringiensis expressed in Novartis Bt 176 maize). The antibody is coupled to an enzyme that catalyzes a color reaction. Within a certain range, the intensity of the color developed on the membrane is proportional to the amount of protein detected by the antibody.

In an ELISA (enzyme-linked immunosorbent assay) the same underlying principle is used but the protein is bound to a well of a plastic plate instead of to a membrane. The plates can have up to 300 wells and most parts of the method can be automated. Western blot is not suitable for routine GM analysis because it is very labor intensive. ELISA offers a number of benefits, such as high throughput and quantitative results when a standard curve is included. Several companies and organizations have developed ELISA GM detection systems and one of them has undergone successful European ring trials for raw and partially processed materials (12).

A significant drawback of ELISA systems is that a transgenic protein may be expressed at low levels or not at all in the part of the plant used in food production. As an example, the Bt toxin is expressed primarily in green parts of Novartis Bt 176 maize; it is expressed only at very low levels in the maize kernels. Therefore, protein detection using antibodies would not be successful in the kernels. In addition, some introduced DNA sequences are not expressed as proteins, as in the transgenic tomato in which only RNA is produced; this RNA inhibits the translation of a protein that influences the ripening process.

EC Regulation 1139/98 (13) states that genetically modified soybeans (covered by EC Decision 96/281/EC) and genetically modified maize (covered by 97/98/EC) have to be labeled if either DNA or protein is present. Consequently, for maize covered by the above decision a DNA analysis has to be performed. From the statement in the open call for the EC Tender XXIV/98/A3/001 (14) it appears that DNA methods for the detection of GM products are preferred by the EC at present. The justification states:

The DNA detection methods have been shown to be more sensitive than the protein detection methods. This is based on preliminary results from research laboratories. To date, no samples have been identified in which protein but no DNA was found, whereas samples containing only DNA and no detectable protein have been identified. This is the reason why this study focuses on DNA detection methods.

In samples where the genetically modified DNA is present, all exogenous DNA may be suitable for detection of GM material (Fig. 2). This includes promoter sequences, the introduced gene itself, endogenous terminator sequences, and marker genes used for selection of transformed organisms. The promoter serves as a start signal to switch on gene expression and production of the protein. In many of the approved plant varieties the 35S promoter derived from the cauliflower mosaic virus (CaMV) is found.

The introduced gene encodes the new protein. The gene can either occur naturally or be fully synthetic (i.e. consist of a sequence of nucleic acids that does not occur naturally and had to be synthesized in a laboratory).

The terminator sequence is the stop signal. The terminator in many of the approved plants is derived from the nopaline synthase gene (NOS) of Agrobacterium tumefaciens, a common soil bacterium. This terminator is called NOS or NOS 3’.

Since during a transformation event fewer than 100% of plants are transformed, scientists need a means of distinguishing the transformed from the nontransformed plants. This can be done by introducing a selective marker gene, for example one coding for resistance to certain antibiotics or to heat. When the plants are grown under the adverse condition to which the marker gene confers protection, only the transformed plants will survive.
Although any of these elements could serve as a target for the detection of genetic modifications, the sequences to be used for this task must be chosen carefully. In some cases sequences due to contamination by bacteria or viruses can be amplified from a food sample. As both the 35S promoter of CaMV and the NOS terminator of A. tumefaciens occur naturally, the presence of one of these sequences is not necessarily evidence for the presence of transgenic material. However, the detection of both sequences in a sample strongly indicates the presence of genetically modified material. For sequences that do not occur naturally the optimal strategy is to amplify overlapping areas comprising the promoter and gene, a sequence arrangement that had to be created in a laboratory. This has been successful for glyphosate-resistant crops (Roundup Ready cotton and soybeans) and insect-resistant Bt maize.

Unlike the ELISA, the PCR is not quantitative. However, two technologies do provide quantitative PCR results: competitive PCR and real-time analysis. Competitive PCR is based on the assumption that if two sequences have the same complementary DNA sequence for the primers to anneal, they will compete for binding of the primers. In this method, a fragment shorter than the target DNA to be amplified in GM plants is synthesized as an internal standard; it contains the same sequence as part of the target, to which the primers may anneal. Since amplification products can be separated by size on an agarose gel and the intensity of their fluorescence is proportional to the amount of DNA present, the amplified DNA from the sample and the internal standard appear on the agarose gel as two distinct bands with different intensities. A dilution series is run to reach the point where the two bands are of equal brightness (Fig. 3). At this point the numbers of target molecules and internal standard molecules are the same. Since the number of internal standard molecules added to the PCR is known, an equal number of target DNA molecules must also be present. This system was demonstrated in a validation study by the Swiss government (15) and further tested in European trials. It is less expensive than the real-time technology but the dilution series is considerably more time consuming.

With the conventional PCR, the band on the agarose gel represents the total number of molecules amplified at the endpoint of the reaction. However, the total number of molecules at the end of a PCR is always roughly the same, irrespective of the number of target sequences present at the beginning. Therefore, it is not possible to obtain quantitative results using PCR in this way. Real-time analysis (also called real-time PCR), in contrast, measures the number of molecules produced during each stage of the PCR rather than just at the end. Since the PCR is a linear function at certain cycles, the number of starting molecules can be calculated by extrapolation. Figure 4 shows a typical reading from a real-time system. Although a real-time system is relatively expensive, it can analyze up to 96 samples at a time.

When a finished product is tested with the described methods, it is not possible to estimate the amount of transgenic material present at the start of the food processing because varying amounts of DNA degradation will have occurred. However, the percentage threshold level introduced by the EU is based on the ingredient level of the food (e.g. maize or soy). The amount of the transgenic component in the food can be estimated by comparing the transgenic component (e.g. 35S promoter), present only in the transgenic portion of soy or maize, to a so-called "household" gene that is present in the transgenic as well as the nontransgenic component. The amount of household gene is set to 100% and the transgenic portion is calculated as a fraction of this. This even works with processed foods, as the transgenic sequence and the household gene sequence are thought to be degraded at the same rate. Therefore, DNA degradation is eliminated from the equation.


The Directorate General Joint Research Centre (DG JRC) has validated a single PCR-based method for the detection of genetically modified products (Roundup Ready soya and Bt 176 maize) and an ELISA method by SDI for Roundup...
Ready soya only (14). The aim of these validation trials was to analyze ground soybeans and maize for the presence of the 35S promoter, NOS terminator, and the Roundup Ready protein. Recently, several qualitative and quantitative (real-time) studies have successfully been performed by DG JRC, the American Association of Cereal Chemists (USA), and the Federal Institute for Health Protection of Consumers and Veterinary Medicine (Germany); and the UK Food Standards Agency has established a proficiency testing scheme that allows assessment of the performance of GMO testing laboratories.

Present and Future of GM-Material Detection

Most laboratories analyze products for the presence of GM material by PCR. However, since several countries have introduced a threshold level above which GM material requires labeling, quantitative methods are increasingly the focus of attention. Real-time analytic methods (ABI Prism 7700 and Light Cycler) are favored; they allow exact calculation of the amount of genetically modified material in a sample. Even so, the problem remains of how to cope with the ever-increasing number of products being approved. If a PCR assay has to be set up for each modification, the analysis will soon become too costly and time consuming. Even if several PCRs are combined (multiplex PCR, where the primer sequences of several traits are in one reaction tube), the number of different sequences that can be amplified simultaneously is usually limited to two or three.

The next anticipated development is the so-called gene-chip, which is already in use in medicine. These chips or multi-array assays have a huge number of DNA sequences bound to defined positions. The sequences, in the case of GM testing, will bind complementary target GM sequences from the sample; these can then be amplified using primers that have a fluorescent tag. The positions of fluorescence on the PCR chip indicate which GM sequences are present in a sample. Such a chip is expected to enter the market momentarily.

Is it thinkable to have, in a few years’ time, a dipstick assay that allows consumers to test goods for GM sequences directly in the supermarket without having to rely on testing laboratories? Thinkable it is, but will GM foods still be an issue in a few years’ time? This will depend on shifts in consumer acceptance and on whether GM crops have shown detrimental effects on the environment or health.

Notes

1. FLAVR SAVR tomato, altered fruit ripening, Calgene, 10/19/1992, Appl. No. 9219601p.
3. TNO, NL; SDI, US.
4. In many cases promoters and terminators originate from bacteria and viruses, e.g. the 35S promoter from cauliflower mosaic virus (CaMV) and the NOS terminator from A. tumefaciens.
5. JRC validates method for the detection of genetically modified soy beans and maize in food raw material. Technical background information at Web site (12) (10-6-98).

Literature Cited