Antibiotic resistance genes and their uses in genetic transformation, especially in plants

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Antibiotic resistance genes and their uses in genetic transformation, especially in plants

General Aspects

Antibiotic resistance as a selection system

Plant biotechnology is based on the delivery, integration and expression of defined genes into plant cells, which can be grown to generate transformed plants. Efficiency of stable gene transfer is not high even in the most successful transfer systems and only a fraction of the cells exposed integrate the DNA construct into their genomes. Moreover, a successful gene transfer does not guarantee expression, even by using signals for the regulation of transgene expression. Therefore, systems to select the transformed cells, tissues or organisms from the non-transformed ones are indispensable to regenerate the truly genetically transformed organisms.

Antibiotic resistance genes allow transformed cells expressing them to be selected for out of populations of non-transformed cells. As part of this system, a selective toxic agent that interferes with the cellular metabolism is applied to a population of putatively transformed cells. The population of cells that has been transformed with and expresses a resistance gene is able to neutralise the toxic effect of the selective agent, either by detoxification of the antibiotic through enzymatic modification or by evasion of the antibiotic through alteration of the target.

The antibiotic resistance genes can be the genes of interest in their own right or they can be operatively linked to other genes to be transformed into the organisms.

Main components of an antibiotic resistance system

The effectiveness of a particular antibiotic resistance system depends mainly on the following elements:

- **The selective agent.** It should fully inhibit growth of untransformed cells. The lowest concentration of the toxic compound that suppresses growth of the non-transformed cells and does not cause detrimental effects to the transformed ones is used.
- **The resistance gene.** Transcriptional and translational control signals fused to the resistance gene determine to a great extent the expression level of resistance. In addition, the gene sequence plays an important role as some are more compatible with animal or plant systems or subgroups of animals and plants, such as monocotyledonous or dicotyledonous plants.
- **The material to be selected.** In the case of plants, sensitivity to the selective agent depends on many factors, including the explant type, the developmental stage, tissue culture conditions and the genotype.
Apart from these factors, for an antibiotic resistance system to be efficient and useful the selectable marker gene should be expressible in a wide variety of cells and tissues, the background metabolic activity or resistance should be minimal or negligible, and a clear phenotypic change should be visible.

The most popular antibiotic resistance marker genes

Among the most widely used antibiotic resistance genes as selectable markers are neomycin phosphotransferase II (nptII) and hygromycin phosphotransferase (hpt). There are also other marker genes like gentamycin acetyltransferase (accC3) resistance and bleomycin and phleomycin resistance, but these are not as commonly used.

The enzyme NPTII inactivates by phosphorylation a number of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (or G418) and paromomycin. Of these, G418 is routinely used for selection of transformed mammalian cells. The other three are used in a diverse range of plant species, however, kanamycin has proved to be ineffective to select legumes and gramineae.

Hygromycin phosphotransferase is a suitable marker system for both plant and animal systems. The HPT enzyme inactivates the antibiotic hygromycin B. Hygromycin is usually more toxic than kanamycin and kills sensitive cells more quickly. It is nowadays one of the preferred antibiotic resistance marker systems for transformation of monocotyledonous plants, particularly gramineae (cereals and forages).

Go to... Current issues on the use of antibiotic resistance genes
Current issues on the use of antibiotic resistance genes

Part of the worldwide debate about genetically-modified organisms focuses on the safety of antibiotic resistance gene markers in crops destined for human and animal consumption. Diverse and, in some cases, contradictory opinions have been voiced. At times, the debate has been unhelpful and polarised. One of the problems is that despite an increasing body of scientific knowledge about genetic modification, the opinions put forward have often been based on perception and emotion, rather than scientific rationale.

In response to these concerns, scientists have focused efforts on identifying potential risks to the ecology, resistance management and food biosafety. The results should allow more informed decisions to be made with respect to this technology.

Engineered antibiotic resistance genes in products for consumption

Several commercial transgenic crops in development or production contain antibiotic resistance genes as part of their new genetic make-up. For instance, crops contain genes whose products confer resistance to kanamycin (the nptII gene), spectinomycin and streptomycin (the aad gene), and ampicillin (the bla gene). Concerns have been raised about whether this may lead to an increase in the occurrence of microbial populations resistant to antibiotics, thereby posing a risk to animal and human health.

Most antibiotic resistance genes used in biotechnology were originally isolated from bacteria. To be used in plants these genes undergo a series of modifications: regulatory elements in the DNA sequence are exchanged for those used in plant cells, and usually the gene sequence is also altered to reflect the preferred codon usage of plants. This would make horizontal gene transfer back to bacteria unlikely.

For further information, a list of recent scientific papers discussing the potential of horizontal transfer of genes from plants to bacteria present in the gut of humans or animals and other related issues has been compiled. The list is neither exhaustive nor comprehensive, but is representative of the research in this area. Click on the link to go to the references.

Alternative methods to antibiotic resistance marker genes

Researchers have devised selection methods that avoid the use of antibiotic or herbicide resistance genes or eliminate them in the final transgenic product. The development of these methods are in part in response to the concerns about horizontal transfer of antibiotic resistance genes, the public perception of risk and the consumer acceptance of the marketed products. Another motivation has been the need for multiple selectable marker genes because the use of a selectable marker gene in a particular line precludes further use of the same selectable gene in subsequent transformations of the same line. The generation of a cultivar with several distinct desirable traits may require repeated transformations events, which would require the use of a different selectable marker for each transformation event. The number of suitable, multiple selectable markers available is limited at present. In addition, the presence of multiple homologous sequences in the same genome may cause instability of the transgenes.

Two general strategies have been pursued to avoid the use of antibiotic resistance genes:
Antibiotic resistance genes and its use in genetic transformation, especially in plants

1. **elimination** of the selectable marker gene in the resultant transgenic organism and
2. use of a **non-toxic compound** that favours or promotes the regeneration and growth of transformed cells expressing a transgene product that acts on the compound (**positive selection**).

Both of these methods and associated IP issues will be discussed in a future white paper.

In the **first strategy**, the methods currently employed are:

- **Co-transformation**. Two separate DNA constructs, one containing a gene of interest and the other having a selectable marker gene are co-transformed into the target cell. As the transformed genes are physically separated, the selectable marker can be eliminated by a variety of mechanisms after assessing the integration and expression of the gene of interest. The system depends on a high efficiency of co-transformation and on the integration of the co-transformed DNAs in distant loci. Co-transformation can be achieved by using either two separate plasmid molecules with the different DNAs in the same transformation agent, e.g. one *Agrobacterium* strain, or by using two different bacterial strains, each containing one of the two constructs.

- **Site-specific recombination systems**. These systems require an enzyme that acts in *trans* (it does not need to be operatively linked to the molecule upon which it acts) to catalyse recombination between two short, cognate DNA sequences. The marker gene, which is cloned between the short cognate sequences, is eliminated when the enzyme, a recombinase, acts upon the sequences. Some of the site-specific recombination systems used are FRT (asymmetric inverted repeat sequences)/FLP (specific recombinase) from *Saccharomyces cerevisiae* and *loxP/Cρe* (causing recombination) from bacteriophage P1.

- **Intra-genomic relocation of transgenes via transposable elements**. Transposition of the maize element families Ac/Ds and the Spm/dSpm is a cut and paste mechanism that results in the excision of the elements of one locus prior to the reinsertion into a second locus. In this way, the selectable marker is removed via transposition of the transposable element that is flanking the marker gene. Failure of the transposable element to reintegrate will cause the selectable marker to be lost.

The **second strategy**, known as **positive selection**, uses selective, non-toxic compounds to exploit the auxotrophies of the transformed material, i.e., the material is unable to regenerate and grow in the absence of an external supply of specific compounds. These methods of positive selection are based on complementing the transformed cells with a gene(s) that enables them

- to produce the essential substance themselves, or
- to be able to utilise a substrate, which confers a metabolic advantage over the non-transformed cells.

Examples of positive selection systems are

- a transgenic glucuronidase in combination with the use of **cytokinin glucuronides** or other phytohormone glucuronides, and
- transgenes such as **phosphomannose isomerase** and **xylose isomerase** that confer the ability to metabolise alternative carbohydrate sources such as mannose and xylose.

Go to... **Patents on antibiotic resistance genes**
References

Assessment of horizontal gene transfer from genetically-modified food into human and animals


Gene flow between transgenic and non-transgenic plants


Traditional plant breeding methods vs. biotechnology


Back to Current issues on the use of antibiotic resistance genes.
Patents on antibiotic resistance genes

Overview

Despite the existence of alternative methods for selection of transgenic organisms, antibiotic resistance genes are still widely used as selectable markers because they are highly efficient, economical and straightforward. Therefore, they are still considered a very valuable tool at experimental and commercial levels. As with many enabling technologies, antibiotic resistance genes are proprietary technologies in the hands of a few entities.

The scope of patent protection ranges from the very broadly claimed use of any antibiotic resistance gene in plant transformation to the more restrictive use of particular antibiotic resistance systems in conjunction with particular promoters and selective agents.

The present paper analyses the extent of patent protection on

- the use of any antibiotic resistance gene, mainly for plant transformation; and
- the most commonly used antibiotic resistance marker genes: neomycin phosphotransferase II (\textit{nptII}) and hygromycin phosphotransferase (\textit{hpt}).

The analysis concentrates on the following aspects

- the entities that have been granted patents or have filed applications in the area;
- the geographical coverage of the patents;
- the nature of the inventions, whether they are products or processes or both, their components and limits;
- comparison between the protected inventions noting overlaps and differences; and
- some implications of dominant patents in this area.

The topics of analysis are

- Antibiotic resistance genes in general;
- Neomycin phosphotransferase gene;
- Hygromycin phosphotransferase gene;
- Dominant patents on antibiotic resistance genes.

Next section... Antibiotic resistance genes in general
Antibiotic resistance genes in general

IP aspects

Monsanto Company holds patent rights on the use of any antibiotic resistance gene as a selectable marker for plant transformation. Importantly, these proprietary rights apply only in the United States and are covered by three granted patents:

- US 5 034 322
- US 6 174 724
- US 6 255 560

These three United States patents are related to three other United States patents and one European patent. However, the other patents are directed to the more specific subject matter of chimeric genes for plant transformation containing the 3SS Cauliflower Mosaic Virus (CaMV) promoter or the promoter of the ribulose−1,5−bis−phosphate carboxylase small subunit (rbcS) gene in combination with the neomycin phosphotransferase (npt) gene as the antibiotic resistance gene. These patents directed to promoters are analysed in the white paper Promoters (available at this web site very soon).

Bibliographic data

<table>
<thead>
<tr>
<th>US 5 034 322</th>
<th>US 6 174 724</th>
<th>US 6 255 560</th>
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</thead>
<tbody>
<tr>
<td>Title</td>
<td>Chimeric genes suitable for expression in plant cells</td>
<td></td>
</tr>
<tr>
<td>Application No. &amp; Filing date</td>
<td>No. 07/333,802 April 5, 1989</td>
<td>No. 08/435,951 May 4, 1995</td>
</tr>
<tr>
<td>Issue date</td>
<td>July 23, 1991*</td>
<td>January 16, 2001**</td>
</tr>
<tr>
<td>Remarks</td>
<td>This patent is related to the US patent 6 174 724 through at least five different applications.</td>
<td>These two patents are only related through the earliest priority document (the first patent application ever filed on the inventions) which corresponds to the United States application 458,414 filed on January 17, 1983.</td>
</tr>
</tbody>
</table>

* Patent term is 17 years from the date of issuance.
** Patent term would be 17 years from the date of issuance, but because of a “terminal disclaimer”, the term does not extend beyond the expiration date of patent US 5 034 322.
† Patent term would be 20 years from the earliest priority date (January 17, 1983), but in this case the term does not extend beyond the expiration date of patent US 5 352 605 (analysed in the white paper on Promoters), which is in 2011.

To view or download the patents as PDF files, click on US 5 034 322 (2.4 kb), US 6 174 724 (2.5 kb) and US 6 255 560 (1.1 kb).

The protected inventions

Plant-expressible promoter

Antibiotic gene

Poly(A)

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Common features

The independent claims of all three United States patents are product claims. There are no method claims.

The product commonly claimed in all three patents is a chimeric construct comprising any antibiotic resistance gene under control of a promoter that works in plants. More specifically, the chimeric genes comprise:

- a promoter region expressible in plant cells,
- a structural DNA sequence encoding a polypeptide that confers antibiotic resistance to the plant cell, and a non–translated region encoding a mRNA polyadenylation signal.

Different features

The independent claims of the United States patents differ from each other in the following aspects:

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<tr>
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</thead>
<tbody>
<tr>
<td>Promoter type</td>
<td><em>A. tumefaciens</em> opine synthase gene &amp; ribulose–1,5–bis–phosphate carboxylase small subunit (<em>rbcS</em> gene) (Claim 1 &amp; 6)</td>
<td>a &quot;naturally expressed&quot; in plants (Claim 1 &amp; 8)</td>
<td>35S CaMV and 19S CaMV (all independent claims)</td>
</tr>
<tr>
<td>Antibiotic resistance gene</td>
<td>any (claim 1) &amp; neomycin phosphotransferase (claim 6)</td>
<td>any (claim 1) &amp; neomycin phosphotransferase (claim 8)</td>
<td>any (all independent claims)</td>
</tr>
<tr>
<td>Plant cell comprising a chimeric gene</td>
<td>not claimed</td>
<td>not claimed</td>
<td>The chimeric gene contains one of the CaMV promoters and any antibiotic resistance gene (Claim 3)</td>
</tr>
<tr>
<td>An intermediate and a final plant transformation Ti plasmid having a chimeric gene</td>
<td>not claimed</td>
<td>not claimed</td>
<td>The intermediate and final Ti plasmid vectors contain a chimeric gene as mentioned above (Claims 6 &amp; 9)</td>
</tr>
<tr>
<td>Particular group of plants having cells containing a chimeric gene</td>
<td>not claimed</td>
<td>not claimed</td>
<td>differentiated dicotyledonous plants (Claim 12)</td>
</tr>
</tbody>
</table>

Analysis of the protected inventions

Definition of terms

Although most of the terms employed throughout the disclosure are clear and correspond to the common usage of the terms in science, the term promoter "naturally expressed" in plants is not expressly defined and, as such, leads to uncertainty about what promoters are covered by these claims. Does "naturally expressed" encompass only promoters from plant genes? or does it include promoters not found as part of plant genes but fully operable in plant cells? The following discussion provides a framework for approaching this issue.

- **What is a promoter "naturally expressed" in plants?**

  The inventors do not provide a precise definition for a promoter "naturally expressed in plants" in the disclosure. The file history of the United States patent 6 174 724, containing all the correspondence held between the U.S. Patent Office and the applicants during the examination process of the application until its issuance, does not reveal the exact scope of the concept either. However, a "guesstimate" of the concept can be drawn from the examples provided in the application and the statements made by the applicants and the examiner during the
examination process.

During examination the patent application was initially rejected on the grounds of being enabling only for the use of the exemplified promoters from the nopaline synthase gene (nos) of the Agrobacterium Ti plasmid and from the ribulose−1,5−bis−phosphate carboxylase small subunit (rbcS) gene, a plant gene. At the time of the invention, 1983, identification, isolation and evaluation of promoters was not a routine task, and the examiner considered that the disclosure did not provide enough guidance for the use of any non−exemplified promoter expressed in plant cells. It appears that the examiner was envisioning promoters expressed in plants from any plant−expressed gene. Although the nos promoter is a promoter of bacterial origin, it was deemed to fall within the concept of plant−expressible promoters as it becomes operational only when the Agrobacterium T−DNA region integrates into the plant cell chromosome and commands the plant cell to initiate the transcription of the nos gene. The applicants finally overcame this ground of rejection by stating that full identification of the regulatory regions of a gene was not an absolute requirement for using the invention. They argued that they had provided a comprehensive method to evaluate the ability of non−exemplified promoters to drive the expression of an antibiotic resistance gene. Notably, the applicants did not disagree with the examiner’s “definition” of naturally expressed promoters.

A further useful insight on the meaning of the term can be gleaned from the applicants' assertion that animal, yeast and bacterial−derived promoters are not plant−expressible promoters and therefore, are not expected to work in plants. They emphasised during the examination process that the promoter should be a plant−expressible promoter capable of functioning in a selected plant cell. The applicants made this assertion in light of experiments conducted by Herrera−Estrella et al. (Nature 303: 209−213, 1983) and Barton et al. (Cell 32: 1033−1043, 1983) on promoters from plant origin and non−plant origin driving heterologous genes in plant cells (see Footnote 1). The exact quote by the applicants in the examination proceedings is as follows:

“For instance, the disclosures in Herrera−Estrella and Barton of the failures of animal, yeast and bacterial genes, i.e. genes containing both coding sequences and promoters from animal, yeast and bacteria, respectively, do not provide evidence of undo experimentation because the present application teaches one to use a plant−expressible promoter capable of functioning in the selected plant cell. This rules out the use of any animal, yeast of bacterial genes as described in the prior art since they do not contain plant−expressible promoters capable of functioning in the selected plant cell. They contain animal, yeast and bacterial promoters which have no expectation of functioning in plant cells. Therefore these teachings do not support that most promoters would fail or provide an expectation that they would fail. These teachings are outside the scope of the present invention since they do not follow the teachings of the present invention—the use of a plant−expressible promoter capable of functioning in the selected plant cell (emphasis added).”


Therefore, a likely interpretation is that a promoter "naturally expressed" in plants may comprise all promoter regions from any plant gene and promoter regions of non−plant origin, which become functional only within a plant cell (e.g. nos).

As with all words, and in the absence of a court's decision, it is difficult to absolutely know the precise limits of "non−plant promoters".

Continued onto... Limitations and implications of the granted patents

1. Herrera−Estrella et al. identified the promoter sequence of the nopaline synthase (nos) gene. They tested its effectiveness by linking it to the octopine synthase (ocs) structural gene and also to the chloramphenicol acetyltransferase coding gene, which confers resistance to the antibiotic chloramphenicol. In both cases, the nos promoter allowed the transcription of the structural genes in plants. On the contrary, the leghaemoglobin gene under the control of its natural promoter sequence did not express in transformed tobacco cells. The leghaemoglobin gene was known to be expressed only in Rhizobium−induced nitrogen fixing nodules of leguminous plants.

Barton et al. cloned the promoter and structural gene of aldehyde dehydrogenase I (Adh−I) from yeast and the promoter and coding sequence of the bacterial gene neomycin phosphotransferase (aph (3')−II) into an Agrobacterium T−DNA and transformed plants with the constructs. There was no expression of the genes.
Limitations and implications of the granted patents

- **Geographical limits**

  It is worth emphasising that the geographical scope of protection for the patented chimeric genes and other features of the inventions is confined to the United States and its territories. Monsanto may enforce its rights over these inventions only in the United States. Users in all other countries are free to employ in any way the products claimed in the patents. The only restriction would come when such users seek to import products, i.e. plants containing chimeric constructs claimed in the patents, into the United States. In this case, users without permission from Monsanto would be in breach of the rights of the patent holder.

- **Any antibiotic resistance and certain promoters as part of the constructs**

  A basic chimeric gene, which is the subject matter of the claims of patents US 5 034 322nd US 6 174 724, corresponds to a chimeric gene similar to those routinely and widely used in plant transformation technologies all over the world. The comprising elements of the chimeric gene of the inventions are the essential components in a chimeric construct designed for the selection of transformed plants based on their acquisition of antibiotic resistance capacity. Thus, essentially, in the United States the use of any antibiotic resistance gene for transformation of plants is pretty much covered by the present Monsanto patents.

  The claims, however broad, do have certain limitations. The antibiotic resistance gene must be used in conjunction with a promoter derived from the opine synthesis genes of A. tumefaciens or from a rbcs gene (‘532 patent); a promoter naturally expressed in plants (‘724 patent) (i.e. promoter from a plant gene or a promoter from a non–plant gene that is normally expressed in a plant cell environment), or the 35S and 19S CaMV promoters (‘560 patent). These limitations potentially leave the window open for the use of promoters from genes of organisms such as bacteria (except maybe Agrobacterium/Rhizobium), fungi, animal and viruses (except CaMV), which are not normally expressed in the plant cells. The inventors themselves ruled out during the examination process promoters of these sorts as part of the scope of their inventions.

- **Any plant or certain plants? A particular vector–transformation method?**

  The United States patent 6 255 560 specifically claims dicot plants having cells containing the chimeric gene (Claim 12). This does not mean, however, that the claimed invention is limited to dicots. Any plant cell comprising a chimeric gene as the one set forth in the patent is covered by the invention (see Claim 3). Since all patents claim as products the described chimeric genes for their expression in plants, virtually any plant containing such products are likely to be encompassed by the claims. The intermediate and final transformation vectors claimed in the United States patent 6 255 560 are specific for Agrobacterium–mediated transformation of plant cells.

- **How such broad claims could be granted so recently?**

  Readers may wonder how patents with such broad claims directed to an enabling technology known and used for selection of transformed plants for at least more than a decade could have been granted at all. In this case, the answer is related to the earliest priority date claimed by the applicants for their inventions. The first filing related to the inventions was January 1983, a time when the development of vectors for transformation and methods to select the transformed cells was taking its first steps. Thus, although the applications for these particular patent documents were filed in 1995 and 1999 (see Bibliography table), the only published
information that counts for assessing the novelty and non-obviousness of the inventions is that published before the earliest priority date claimed: that is, the relevant prior art to the inventions published prior to January 17, 1983. The myriad research and developments in this area published after January 17, 1983 do not count for assessing patentability of the inventions.

View Independent Claims

Back to Overview

Next section... Neomycin phosphotransferase gene
Antibiotic resistance genes in general

Patents granted to Monsanto

Actual granted independent claims

<table>
<thead>
<tr>
<th>US 5 034 322</th>
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<tbody>
<tr>
<td><strong>Claim 1</strong></td>
</tr>
<tr>
<td>A chimeric gene capable of expressing a polypeptide in plant comprising in sequence:</td>
</tr>
<tr>
<td>a) a promoter region from a gene selected from the group consisting of an <em>Agrobacterium tumefaciens</em> opine synthase gene and a ribulose−1,5−bis−phosphate carboxylase small subunit gene;</td>
</tr>
<tr>
<td>b) a structural DNA sequence encoding a polypeptide that permits the selection of transformed plant cells containing said chimeric gene by rendering said plant cells resistant to an amount of an antibiotic that would be toxic to non−transformed plant cells, said structural DNA sequence being heterologous with respect to the promoter region; and</td>
</tr>
<tr>
<td>c) a 3' non−translated region of a gene naturally expressed in plants, said region encoding a signal sequence for polyadenylation of mRNA.</td>
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<tr>
<th>US 6 174 724</th>
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<tbody>
<tr>
<td><strong>Claim 1</strong></td>
</tr>
<tr>
<td>A chimeric plant−expressible gene, said gene comprising in the 5' to 3' direction:</td>
</tr>
<tr>
<td>a) a promoter region derived from a gene that is naturally expressed in a plant cell and that is capable of effecting mRNA transcription in the selected plant cell to be transformed, operably linked to</td>
</tr>
<tr>
<td>b) a structural DNA sequence encoding a polypeptide that permits the selection of transformed plant cells containing said chimeric gene by rendering said transformed plant cells resistant to an amount of an antibiotic that would be toxic to non−transformed plant cells, operably linked to</td>
</tr>
<tr>
<td>c) a non−translated region of a gene naturally expressed in plant cells, said region encoding a signal sequence for polyadenylation of mRNA.</td>
</tr>
</tbody>
</table>
Claim 8

A chimeric gene capable of expressing a polypeptide in plant cells comprising in sequence:

a) a promoter region from a gene which is naturally expressed in plant cells;

b) a 5' non−translated region;

c) a structural coding sequence encoding a neomycin phosphotransferase polypeptide; and

d) a 3' non−translated region of a gene naturally expressed in plant cells, said region encoding a signal sequence for polyadenylation of mRNA.

Claim 1

A chimeric gene which is expressed in plant cells comprising:

a) a promoter from cauliflower mosaic virus (CaMV), wherein said promoter is the CaMV(19S) promoter or the CaMV(35S) promoter, operably linked to a DNA sequence which is heterologous with respect to the promoter, wherein:

i. the promoter regulates the transcription of the DNA sequence, and

ii. the DNA sequence encodes a polypeptide conferring increased antibiotic resistance to a plant or plant cell containing the DNA sequence relative to a wild−type plant or plant cell

Claim 3

A plant cell comprising a chimeric gene which comprises:

a) a promoter from cauliflower mosaic virus (CaMV), wherein said promoter is the CaMV(19S) promoter or the CaMV(35S) promoter, operably linked to a DNA sequence which is heterologous with respect to the promoter, wherein:

i. the promoter regulates the transcription of the DNA sequence, and

ii. the DNA sequence encodes a polypeptide conferring increased antibiotic resistance to the plant cell relative to a wild−type plant cell.

Claim 6

An intermediate plant transformation plasmid which comprises:

a) a region of homology to an A. tumefaciens vector;

b) a T−DNA border from A. tumefaciens, and

c) a chimeric gene,

wherein the chimeric gene is located between the T−DNA border and the region of homology, said chimeric gene comprising a promoter from cauliflower mosaic virus (CaMV), wherein said promoter is the CaMV(19S) promoter or the CaMV(35S) promoter, operably linked to a DNA sequence which is heterologous with respect to the promoter, wherein:

a) the promoter regulates the transcription of the DNA sequence, and

b) the DNA sequence encodes a polypeptide conferring increased antibiotic resistance to a plant or plant cell containing the DNA sequence relative to a wild−type plant or plant cell.
Claim 9
A plant transformation vector which comprises a modified plant tumor inducing plasmid of *A. tumefaciens* which is capable of inserting a chimeric gene into susceptible plant cells, wherein the chimeric gene comprises a promoter from cauliflower mosaic virus (CaMV), wherein said promoter is the CaMV(19S) promoter or the CaMV(35S) promoter, operably linked to a DNA sequence which is heterologous with respect to the promoter, wherein:

a) the promoter regulates the transcription of the DNA sequence, and
b) the DNA sequence encodes a polypeptide conferring increased antibiotic resistance to a plant or plant cell containing the DNA sequence relative to a wild-type plant or plant cell.

Claim 12
A differentiated dicotyledonous plant comprising plant cells containing a chimeric gene which comprises a promoter from cauliflower mosaic virus (CaMV), wherein said promoter is the CaMV(19S) promoter or the CaMV(35S) promoter, operably linked to a DNA sequence encoding said polypeptide which is heterologous with respect to the promoter, wherein:

a) the promoter regulates the transcription of the DNA sequence, and
b) the DNA sequence encodes a polypeptide conferring increased antibiotic resistance to the plant relative to a wild-type plant.
Neomycin phosphotransferase (npt) gene

General aspects

Scientific info

Two neomycin phosphotransferase genes are used in selection of transformed organisms: the neomycin phosphotransferase I (nptI) gene and the neomycin phosphotransferase II (nptII) gene. The second one is the more widely used. It was initially isolated from the transposon Tn5 that was present in the bacterium strain *Escherichia coli* K12. The gene codes for the aminoglycoside 3′−phosphotransferase (denoted APH(3′)−II or NPTII) enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as:

- kanamycin
- neomycin
- geneticin (G418), and
- paromomycin.

NPTII is probably the most widely used selectable marker for plant transformation. It is also used in gene expression and regulation studies in different organisms in part because N−terminal fusions can be constructed that retain enzymatic activity. In animal cells, G418 and neomycin are used as selectable agents.

NPTII protein activity can be detected by enzymatic assay. In other detection methods, the modified substrates –the phosphorylated antibiotics– are detected by thin−layer chromatography, dot−blot analysis or polyacrylamide gel electrophoresis.

Plants such as maize, cotton, tobacco, *Arabidopsis*, flax, soybean and many others have been successfully transformed with the nptII gene. In plants, kanamycin is the most commonly used selective agent, normally in concentrations ranging from 50 to 500 mg/l. It is very effective in inhibiting the growth of untransformed cells. However, kanamycin is ineffective as a selection marker for several legumes and gramineae. For instance, in rice, kanamycin seems to interfere with the regeneration of transformed cells to green plants. As an alternative, paromomycin can be used for selecting nptII−transformed rice cells. Therefore, the choice of the selective agent is important and based on the plant species to be transformed.

Agroindustrial applications

Field trials and commercial releases

According to information provided by BioTrack, a database administered by the Organisation for Economic Cooperation and Development (OECD) containing records of field trials and commercial releases in OECD countries (currently 30) from 1996 to 2000, essentially all of genetically−modified organisms (GMO) are plants (98.4%). Most of the research and
development of GMOs is carried out in the United States (71.1%). The rest of the OECD countries contribution to GMOs is less than 10% each, with Canada close to 9% and the other countries ranging between 5% and 0.6%. Among plants, maize is the crop with the largest number of genetically-modified varieties (37.4%) followed by oilseed rape (12.4%) and potato (12.1%).

Most introduced traits in the modified crops confer resistance to compounds such as herbicides, pests, such as insects and nematodes, and diseases caused by bacteria, fungi and viruses. Characteristics such as colour of flowers, delayed ripening of fruits, and sterility have also been introduced in plants to a lesser extent. Antibiotic resistance is not a trait of interest for most of the modified plants. Nevertheless, nptII is a feature present in many plant releases because it has been used to assist in their selection.

According to the information on globally approved GM plants compiled and provided by Agriculture & Biotechnology Strategies (Canada) Inc., modified plants containing nptII gene that are approved for release into the environment as food or feed products include maize, canola (oilseed rape), melon, potato, tomato and cotton as a fiber crop. Most of the releases have occurred in the United States. However, some transformed cotton varieties developed by Monsanto have been approved in several other countries such as Australia, Argentina, Canada, China, India, Japan, Mexico and South Africa.

Multiple risk assessments of crops, including those for human consumption, containing the nptII gene and its protein have found that there are no scientific reasons to deny or restrict the use of this gene in transgenic crops on grounds of human, animal or environmental safety.

Go to... IP aspects
IP aspects of the \textit{npt} gene

Analysis of the filed and granted inventions

The selected analysed patents and patent applications related to the \textit{npt} gene(s) are divided as follows:

- \textit{npt} gene as part of a chimeric gene construct for plant transformation
- \textit{nptII} gene in combination with paromomycin as a selective agent
- \textit{nptII} gene as part of a bifunctional marker gene
- \textit{Aminoglycoside phosphotransferase} gene conferring resistance to G418

- \textit{npt} gene as part of a chimeric gene construct for plant transformation.

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<thead>
<tr>
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<tbody>
<tr>
<td><strong>Title</strong></td>
<td>Chimeric genes suitable for expression in plant cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Application No. &amp; date</td>
<td>No. 08/435,951 May 4, 1995</td>
<td>No. 07/ 333,802 April 5, 1989</td>
<td>EP 84900782.8 January 16, 1984</td>
</tr>
<tr>
<td>Issue date</td>
<td>January 16, 2001*</td>
<td>July 23, 1991**</td>
<td>July 28, 1999*</td>
</tr>
<tr>
<td>Language</td>
<td>English</td>
<td>English</td>
<td>English (Claims in English, German and French)</td>
</tr>
<tr>
<td>Remarks</td>
<td>The two United States patents are related to the European patent only through the earliest priority document (the first patent application ever filed on the inventions), which corresponds to the United States application 458,414 filed on January 17, 1983. * Patent term would be 17 years from the date of issuance, but because of a “terminal disclaimer”, the term does not extend beyond the expiration date of patent US 5 034 322. ** Patent term is 17 years from the date of issuance. *The European patent was initially granted on March 6, 1991. As a result of an opposition filed against it by several entities, the scope of the claimed invention was modified. The amended claims and description were published eight years later on the date given. Patent term is 20 years from the date of filing the application.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To view or download the patents as PDF files, click on US 6 174 724 (2.5 kb), US 5 034 322 (2.4 kb) and EP 131 623 B2 (2.8 Mb).
Monsanto has two granted United States patents and one granted European patent claiming chimeric constructs for plant transformation containing a gene encoding a neomycin phosphotransferase enzyme, which confers antibiotic resistance to the transformed plant. The chimeric constructs of the inventions also comprise a promoter and a polyadenylation signal sequence. The United States patents 6 174 724 and 5 034 322 are also analysed in Antibiotic resistance genes in general.

One of the limitations of the inventions lies in the sort of promoter used in the chimeric construct to control the npt gene:

<table>
<thead>
<tr>
<th>Promoter type</th>
</tr>
</thead>
<tbody>
<tr>
<td>US 6 174 724</td>
</tr>
<tr>
<td>Promoter type</td>
</tr>
<tr>
<td>US 5 034 322</td>
</tr>
<tr>
<td>EP 131 623 B2</td>
</tr>
</tbody>
</table>

Promoters may originate from genes of plant or other organism origins.

As discussed in the section, Antibiotic resistance genes in general, a promoter "naturally expressed" in plants is not explicitly defined by the inventors. Yet the meaning of the term can be deduced from the description of the invention and the file history of the patent. Both sources tell us that the inventors may have envisioned any promoter from a gene of plant origin and promoters from genes of other organisms such as the nopaline synthase (nos) gene of A. tumefaciens, which is expressed only in a plant cell under natural conditions.

Opine genes are present in the Ti (tumour–inducing) plasmids and Ri (root–inducing) plasmids of Agrobacterium species. These genes are inactive while in the bacterial cells and are expressed only after they enter the plant cells. They code for enzymes that metabolise substances called "opines," such as octopine, nopaline, and agropine. Opines are utilized by the bacteria as a source of carbon, nitrogen, and energy. The promoter claimed in the US patent 5 034 322 can be derived from any of the opine genes present in the Ti plasmids of the species A. tumefaciens.

Ribulose–1,5–bis–phosphate carboxylase (Rbc) catalyzes the reduction of atmospheric CO2 during photosynthesis. In higher plants, Rbc is a protein composed of eight copies of chloroplast–encoded large subunits and eight copies of nuclear–encoded small subunits (ss). The promoter claimed in the United States patent 5 034 322 and the European patent is isolated from a gene encoding a small subunit. There is no limitation on the plant source of the gene; it can be derived from any plant.

The antibiotic resistance gene coding for neomycin phosphotransferase is not restricted to a particular gene sequence. The protected gene in the European patent codes for either a neomycin phosphotransferase I or neomycin phosphotransferase II. According to the inventors, these are distinct enzymes with major differences in their amino acid sequences and substrate specificity. Thus, in the United States and in Europe, chimeric constructs designed for plant cells having any DNA sequence encoding a neomycin phosphotransferase could be encompassed by the claims.
Japan Tobacco has filed a European patent application directed to the use of the \textit{nptII} gene in combination with the antibiotic \textit{paromomycin} for the selection of transformed rice cells.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{paromomycin.png}
\caption{Structure of paromomycin antibiotic}
\end{figure}

The \textit{nptII} gene as a selectable marker for monocot plants, especially rice, has not been very effective when used in combination with kanamycin as a selective agent. This antibiotic negatively affects the regeneration of the transformed plants. The performance of G418 as a selective agent for monocots is better, but there is still poor transformation efficiency. The applicants claim that the combination of the \textit{nptII} gene with paromomycin constitutes a highly efficient rice transformation system.

The filed claims of the European patent application are limited to:

\begin{itemize}
\item a method to \textbf{select} transformed rice cells, which contain a gene of interest and a \textit{paromomycin resistance gene} by applying paromomycin to the medium;
\item a method for \textbf{producing} rice transformants by \textit{Agrobacterium}–mediated transformation of rice cells with a construct having a gene of interest and a \textit{paromomycin resistance gene}. The transformed cells are selected with paromomycin and regenerated into complete plants; and
\item transformed rice selected following the method mentioned above.
\end{itemize}

The paromomycin resistance gene is not limited to the \textit{nptII} gene in the filed independent claims. Thus, the invention might encompass any gene that confers resistance to paromomycin, including the \textit{nptII} gene. The use of paromomycin and a paromomycin resistance gene for the selection of rice plants is one of the main limitations of the invention as filed. It is not possible to ascertain the exact limitations of the claims as the application has not been granted yet.
Antibiotic resistance genes and its use in genetic transformation, especially in plants

Continued onto...

- *nptII* gene as part of a bifunctional marker gene
- *Aminoglycoside phosphotransferase* gene conferring resistance to G418
The National Research Council of Canada has two granted patents, in the United States and in Europe, directed to dual genetic markers composed of fused genes, which provide a reporter marker gene (glucuronidase (gusA) gene) and an antibiotic resistance gene (nptII gene).

The gusA gene encodes β−glucuronidase (GUS), a hydrolase that cleaves a wide variety of β−glucuronides. GUS is the most widely used reporter system for plants. It is easy to quantify, highly sensitive and very specific. Substrates for GUS are available for spectrometric, fluorometric and histochemical detection assays.

The bifunctional genetic marker of the invention allows for genetic selection of the transformed cells (nptII gene) and subsequent spatial localisation and quantitative estimation of gene activity (gus gene).

The invention is limited with respect to the components of the fusion marker. However, the host organism expressing the marker is not limited to any organism in particular. It could potentially be any host as long as it is capable of expressing the stable polypeptide having both activities.

View Independent Claims

**Aminoglycoside phosphotransferase gene conferring resistance to G418**

![Structure of G418 antibiotic](image)

There are several aminoglycoside phosphotransferases conferring resistance to aminoglycoside antibiotics. The aminoglycoside phosphotransferase I (APH−I) enzyme and the aminoglycoside (or neomycin) phosphotransferase II (APH−II or NPTII) are unrelated except for their ability to inactivate the
Antibiotic resistance genes and its use in genetic transformation, especially in plants

antibiotic G418. The *aph*–I gene was originally found on transposon Tn601, also known as Tn903. According to some reports, APH–I is approximately four times more effective than APH–II in inactivating G418.

Cetus Corporation (acquired by Hoffman–La Roche in the early 90’s) has two granted patents, one in the United States and one in Canada, on the DNA sequence of a modified APH–I enzyme. Modified truncated *aph*-I gene can be used as a selectable marker for both prokaryotic and eukaryotic organisms.

<table>
<thead>
<tr>
<th>US 4 784 949</th>
<th>CA 1337716 A1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Title</strong></td>
<td>Universal dominant selectable marker cassette</td>
</tr>
<tr>
<td><strong>Application No. &amp; Filing date</strong></td>
<td><strong>Issue date</strong></td>
</tr>
<tr>
<td>US 602118 April 19, 1984</td>
<td>November 15, 1988*</td>
</tr>
<tr>
<td>CA 475153 A February 26, 1985</td>
<td>December 12, 1995*</td>
</tr>
<tr>
<td><strong>Remarks</strong></td>
<td>The related granted U.S. patent 5 116 750 directed to a fusion protein containing APH–I enzyme expired due to lack of payment of fees on September 26, 2000.</td>
</tr>
<tr>
<td></td>
<td>*The patent term of both the United States and the Canadian patents is 17 years from the date of issuance.</td>
</tr>
</tbody>
</table>

To view or download the patent as a PDF file, click on [US 4 784 949](2.5 kb).

Both patents claim a truncated DNA sequence of an *aph*-I gene, which contains restriction sites immediately upstream of the start codon (Claim 1). This set-up ensures a precise reproducible translation of the gene and permits the construction of fusion proteins that contain the APH–I sequences at the C–terminal end. The *aph*-I gene of the invention is further modified by removing the codons for amino acids in positions 2–10, inclusive, and a couple of restriction sites within the codifying region. This modified truncated *aph*-I (mt *aph*-I) gene is particularly effective against G418. It also inactivates the antibiotic neomycin effectively, but it is less effective against kanamycin than the *nptII* (or *aph*-II) gene.

The Canadian patent further claims:

- a recombinant expression vector having the mt *aph*-I gene capable of conferring resistance to G418 on prokaryotes and eukaryotes (Claim 4);  
- an expression vector with the mt *aph*-I gene as described under the control of promoter for eukaryotic cells (Claim 17);  
- an expression vector for eukaryotes having a sequence for a fusion protein that has a mt *aph*-I gene as described in its C–terminal (Claim 19);  
- a fusion protein that contains a C–terminal mt *aph*-I sequence and a N–terminal sequence of:  
  1. any desired protein (Claim 30);  
  2. ß–isopropylmalate dehydrogenase (Claim 31); and  
  3. yeast enolase (Claim 35).  
- a method of purifying a fusion protein that comprises the mt *aph*-I gene as described (Claim 37).

There may be some marginal overlap between the United States patents filed by Monsanto and the United States patent granted to Cetus Corporation. Although Monsanto’s patents are directed to chimeric genes, the DNA sequence coding for a neomycin phosphotransferase is not limited to any in particular. So, it might well include a neomycin phosphotransferase I (*aph*-I) gene or a neomycin phosphotransferase II (*nptII* or *aph*-II) gene.

The sequence claimed by Cetus Corporation has some limitations. It is a modified and truncated version of *aph*-I. Thus, it is likely that an *aph*-I gene without the modifications of the claimed version of the gene would not be encompassed by the claims.

View Independent Claims
Antibiotic resistance genes and its use in genetic transformation, especially in plants

Back to... Overview.

Next section... Hygromycin phosphotransferase gene.
Neomycin phosphotransferase gene

1. \textit{npt} gene as part of a chimeric gene construct for plant transformation

Patents granted to Monsanto

Actual granted independent claims

<table>
<thead>
<tr>
<th>US 5 034 322</th>
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<tbody>
<tr>
<td><strong>Claim 6</strong></td>
</tr>
<tr>
<td>A chimeric gene comprising in sequence:</td>
</tr>
<tr>
<td>A) a promoter region from a gene selected from the group consisting of an \textit{Agrobacterium tumefaciens} opine synthase gene and a ribulose−1,5−bis−phosphate carboxylase small subunit gene;</td>
</tr>
<tr>
<td>B) a heterologous structural DNA sequence encoding a neomycin phosphotransferase; and a 3' non-translated C) region of a gene naturally expressed in plant cells, said region encoding a signal sequence for polyadenylation of mRNA.</td>
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</table>

<table>
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<tr>
<th>US 6 174 724</th>
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<tbody>
<tr>
<td><strong>Claim 8</strong></td>
</tr>
<tr>
<td>A chimeric gene capable of expressing a polypeptide in plant cells comprising in sequence:</td>
</tr>
<tr>
<td>A) a promoter region from a gene which is naturally expressed in plant cells;</td>
</tr>
<tr>
<td>B) a 5' non-translated region;</td>
</tr>
<tr>
<td>C) a structural coding sequence encoding a neomycin phosphotransferase polypeptide; and</td>
</tr>
<tr>
<td>D) a 3' non-translated region of a gene naturally expressed in plant cells, said region encoding a signal sequence for polyadenylation of mRNA.</td>
</tr>
</tbody>
</table>

2. \textit{nptII} gene in combination with paromomycin as a selective agent

Patent filed by Japan Tobacco

Actual filed independent claims

<table>
<thead>
<tr>
<th>EP 927 765 A1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Claim 1</strong></td>
</tr>
<tr>
<td>A method for selecting transformed cells which comprises:</td>
</tr>
<tr>
<td>A) culturing cells originating from rice tissue in a selective medium containing paromomycin, after transferring at least a desired structural gene and a paromomycin resistance gene into the cells, and</td>
</tr>
<tr>
<td>B) selecting transformed cells.</td>
</tr>
</tbody>
</table>

**Claim 7**
A transformed rice cell which is selected from cells originating from rice tissue by culturing the cells in a selective medium containing paromomycin after at least a desired structural gene and a paromomycin resistance gene were transferred into the cells.
Claim 8
A method for producing a rice transformant transformed by a desired gene comprising:

A) providing a strain belonging to the genus *Agrobacterium* which has a plasmid containing a paromomycin resistance gene and a desired gene, in the T−DNA region in said plasmid, in such a way as to allow expression of each of said genes;
B) providing cells originating from rice tissue;
C) inoculating the above cells originating from rice with the above strain belonging to the genus *Agrobacterium*;
D) culturing the inoculated cells in a culture medium for plant cells containing paromomycin at such a concentration that will not allow cells other than those having been transformed by the paromomycin resistance gene to survive in said medium (hereinafter referred to as a selective medium), and selecting paromomycin−resistant calluses;
E) if necessary, repeating the selection step d) once or more by using tissue cells obtained from the selected calluses; and
F) culturing the selected calluses in a medium appropriate for plant regeneration to give a completely regenerated plant.

3. *nptII* gene as part of a bifunctional marker gene

*Patents granted to the National Research Council of Canada*

*Actual granted independent claims*

<table>
<thead>
<tr>
<th>US 5 639 663</th>
</tr>
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<tbody>
<tr>
<td><strong>Claim 1</strong></td>
</tr>
<tr>
<td>A fused gene comprising a first structural gene which encodes beta−glucuronidase activity, fused in frame and linked by an intergenic nucleotide sequence to a second structural gene which encodes neomycin phosphotransferase−II activity, and in a suitable host is capable of expressing a single, stable polypeptide translation product simultaneously having the combined activities of the first and second structural genes.</td>
</tr>
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</table>

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<thead>
<tr>
<th>EP 583 258 B1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Claim 1</strong></td>
</tr>
<tr>
<td>A nucleotide which comprises beta−glucuronidase and neomycin phosphotransferase−II structural genes fused in frame and linked by an intergenic nucleotide sequence, wherein the nucleotide encodes and, in a suitable host is capable of expressing a single stable polypeptide translation product having both beta−glucuronidase and neomycin phosphotransferase−II activities.</td>
</tr>
</tbody>
</table>
4. Coding sequence for aminoglycoside phosphotransferase I (APH−I)

Patents granted to Cetus Corporation (U.S. patent) & Chiron Corporation (CA patent)

Actual granted independent claims

<table>
<thead>
<tr>
<th>US 4 784 949* &amp; CA 1337716 A1</th>
</tr>
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<tbody>
<tr>
<td>(<strong>Only Claim 1</strong>)</td>
</tr>
</tbody>
</table>

**Claim 1**
A DNA sequence cassette encoding a dominant selectable marker effective in prokaryotic and eukaryotic transformants which comprises:

a) an ATG start codon in reading frame with the codons of a modified truncated aminoglycoside phosphotransferase−I (aph−I) gene, designated herein mtaph−I, and
b) at least one cassette−unique restriction site upstream of and proximal to the ATG start codon,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of said cassette−unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.

**Claim 4**
A recombinant expression vector capable of conferring resistance to G418 on a prokaryotic or eukaryotic transformant which vector comprises:

- an ATG start codon in reading frame with the codons of the mtaph−I gene, all operably linked to appropriate control sequences functional in the host harboring the vector,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of a cassette−unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.

**Claim 17**
An expression vector, operable in eukaryotic host cells, which comprises:

- mtaph−I coding sequences operably linked to control sequences functional in the eucaryotic host,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of a cassette−unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.
Claim 19
An expression vector, operable in eucaryotic host cells, which comprises:

- a coding sequence for a fusion protein operably linked to control sequences functional in the eucaryotic host,
  wherein the encoded fusion protein comprises:
  
  a) a desired peptide as the N-terminal sequence and
  b) mtaph−I as the C-terminal sequence,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of a cassette–unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.

Claim 30
A fusion protein which comprises:

a) an N-terminal sequence comprising the N-terminal amino acid sequence of a desired protein and
b) a C-terminal sequence comprising mtAPH−I,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of a cassette–unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.

Claim 31
A fusion protein which comprises:

a) an N-terminal sequence comprising the N-terminal amino acid sequence of B-isopropylmalate dehydrogenase and
b) a C-terminal sequence comprising mtAPH−I,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of a cassette–unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.

Claim 35
A fusion protein which comprises:

a) an N-terminal sequence comprising the N-terminal amino acid sequence of yeast enolase and
b) a C-terminal sequence comprising mtAPH−I,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of a cassette–unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.
Claim 37
A method of purifying a fusion protein which method comprises:

- subjecting a fusion protein containing mtAPH−I to an amino glycoside containing support,

said mtaph−I defined herein as the coding sequence for APH−I modified so as to retain the amino acid sequence encoded by the aph−I gene, but to eliminate any restriction site otherwise duplicated of said cassette–unique restriction site, and truncated by deletion of codons encoding at least one amino acid in positions 2 – 10 inclusive.
Hygromycin phosphotransferase (*hpt*) gene

**General aspects**

**Scientific info**

The hygromycin phosphotransferase (denoted *hpt*, *hph* or *aph* IV) gene was originally derived from *Escherichia coli*. The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin B. A large number of plants have been transformed with the *hpt* gene and hygromycin B has proved very effective in the selection of a wide range of plants, including monocotyledonous. Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals. Likewise, the *hpt* gene is used widely in selection of transformed mammalian cells.

![Structure of Hygromycin B antibiotic](image)

The sequence of the *hpt* gene has been modified for its use in plant transformation. Deletions and substitutions of amino acid residues close to the carboxy (C)−terminus of the enzyme have increased the level of resistance in certain plants, such as tobacco. At the same time, the hydrophilic C−terminus of the enzyme has been maintained and may be essential for the strong activity of HPT.

HPT activity can be checked using an enzymatic assay. A non−destructive callus induction test can be used to verify hygromycin resistance. The antibiotic hygromycin B should be handled with care because it is toxic to humans.

**Agroindustrial applications**

**Field trials and commercial releases**

Like the *nptII* marker gene, *hpt* has been used as a selectable marker gene for transgenic plants, but it is not an agronomic trait of interest in modified plants. It is one of the introduced genes needed to accomplish the production of transformed plants.

According to information provided by BioTrack, a database administered by the Organisation for Economic Cooperation and Development (OECD) containing records of field trials and commercial releases in OECD Member countries (currently 30), several cultivars of rapeseed, alfalfa and canola submitted to field trials in the United States and Canada between 1989 and 1996 contained hygromycin phosphotransferase as a selectable marker gene.
In Australia, several varieties of barley, wheat, grapevine, indian mustard and poppy, engineered for viral tolerance, fungal resistance, improvement of fruit quality and insect resistance contain a hygromycin phosphotransferase gene. These crops are being tested in field trials and have not been commercially released yet.
IP aspects of the *hpt* gene

Analysis of the protected inventions

The patents on the *hpt* gene and its applications in prokaryotic and eukaryotic transformation are a very interesting example of a comprehensive patent protection strategy followed by a company to consolidate an exclusive position in an enabling tool around the world.

**Eli Lilly & Company** has 22 granted patents (as of July 2002) in at least 10 different countries and a couple of patent applications that cover:

1. the wild-type, isolated *hpt* gene and vectors containing it for prokaryotic and eukaryotic expression;
2. a modified *hpt* gene; and
3. plasmids containing a modified *hpt* gene for plant transformation.

The patents granted in the **United States**, **Canada** and **Germany** have been assigned to **Novartis** (now **Syngenta**).

The patents are divided into three families according to their common priority applications and are directed to the following aspects:

**Family 1. Recombinant DNA cloning vectors having the *hpt* gene for eukaryotic and prokaryotic cell transformation**

**Family 2. Modified hygromycin B resistance–conferring gene**

**Family 3. Selectable marker for development of vectors and transformation systems in plants**

**Family 1. Recombinant DNA cloning vectors having the *hpt* gene for eukaryotic and prokaryotic cell transformation**

**Eli Lilly**'s portfolio of patents on this matter starts with this group, which discloses recombinant DNA cloning vectors that confer resistance to hygromycin (hyg B) and G418 antibiotics in both prokaryotic and eukaryotic cells. The patents encompass the initially isolated gene sequences for these antibiotic resistance genes.

The gene coding for an enzyme conferring resistance to G418 **does not** correspond to the *nptI* nor the *nptII* gene from the transposons Tn603 and Tn5, respectively. In this case it is a gene encoding an aminoglycoside acetyltransferase enzyme.

A cloning vector of the invention comprises:

1. a eukaryotic promoter;
2. structural gene(s) and its associated control sequence(s) for either hyg B or G418 resistance or both; and
3. a prokaryotic replicon.

When the host cell is a prokaryote, the antibiotic resistance genes and their control sequences are adjacent to the prokaryote replicon. In the case of a eukaryotic host cell, the eukaryotic promoter drives a **single** gene, either *hpt* or G418 resistance gene, but not both.

The plasmid pKC203 from the *Escherichia coli* JR225 strain is the parent plasmid harboring both antibiotic conferring–resistance genes used for the construction of a series of plasmids for use in transformation of prokaryotic and eukaryotic cells.

<table>
<thead>
<tr>
<th>Country</th>
<th>Granted Patent No.</th>
<th>Filing date</th>
<th>Issue date</th>
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<tbody>
<tr>
<td>Australia</td>
<td>AU 555 574 B2</td>
<td>June 17, 1982</td>
<td>October 2, 1986</td>
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<tr>
<td></td>
<td>AU 582 653 B2</td>
<td>May 27, 1986</td>
<td>April 6, 1989</td>
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<tr>
<td>Canada</td>
<td>CA 1195626 A1</td>
<td>June 16, 1982</td>
<td>October 22, 1985*</td>
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Antibiotic resistance genes and its use in genetic transformation, especially in plants

<table>
<thead>
<tr>
<th>Country</th>
<th>Patent No.</th>
<th>Priority Date</th>
<th>Application Date</th>
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<tbody>
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<td>Denmark</td>
<td>DK 172716 B1</td>
<td>June 16, 1982</td>
<td>June 14, 1999</td>
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<tr>
<td>Europe**</td>
<td>EP 68740 B1</td>
<td>June 17, 1982</td>
<td>March 22, 1989</td>
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<td>Great Britain</td>
<td>GB 2146031</td>
<td>September 27, 1984</td>
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<td>Hungary</td>
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<td>June 17, 1982</td>
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<td>Ireland</td>
<td>IE 8853521 B</td>
<td>June 17, 1982</td>
<td>December 7, 1988</td>
</tr>
<tr>
<td>Former USSR</td>
<td>SU 1250174 A3</td>
<td>June 16, 1982</td>
<td>August 7, 1986</td>
</tr>
<tr>
<td>United States</td>
<td>US 4 727 028</td>
<td>September 30, 1983</td>
<td>February 23, 1988*</td>
</tr>
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</table>

*Patent term of the Canadian and United States patents is 17 years from the date of issuance.

**The European patent was converted to a national patent in Belgium (BE), France (FR), Great Britain (GB), Germany (DE), Italy (IT); Lichtenstein (LI); Luxemburg (LU); Netherlands (NL), Sweden (SE) and Switzerland (CH).

There are related patent applications pending in Greece, Israel and Japan.

To view or download the patents as PDF files, click on EP 68740 B1 (2.0 kb) and US 4 727 028 (2.4 kb).

The plasmid pKC2O3 is the source of restriction fragments containing both or either one of the antibiotic resistance genes, which are then inserted into different plasmids. This series of plasmids are part of the claimed invention. The claimed fragments are:

- a 7.5 kb Bgl II fragment containing both resistance genes;
- a 2.5 kb Bgl II/Sal I fragment containing both resistance genes;
- a 1.51 kb Sac I/Bgl II fragment containing the *hpt* gene; and
- a 1.61 kb EcoR I/Sal I fragment containing the G418 resistance gene.

The antibiotic resistance genes of one of the recombinant cloning vectors are claimed in general terms without defining a specific DNA sequence (Claim 1 of the United States and Canadian patents, and Australian patent 555 574 B). In these countries, the invention is likely to cover any DNA sequence encoding the enzymes against these antibiotics.

Transformed prokaryotic and eukaryotic host cells are also the subject of independent claims. Eukaryotic host cells include mouse, *Escherichia coli*, *Saccharomyces cerevisiae* and human. It does not mean, however, that the recombinant vectors of the invention are only limited to this group of hosts. Other independent claims encompass eukaryotes and
prokaryotes in general. Thus, virtually any organism could be covered by the invention.

All the above mentioned features are the subject matter of the Australian, Canadian, European and United States patents. In addition, the United States patent claims the amino acid sequence of the hygromycin phosphotransferase (HPT) enzyme. Patents granted in other countries were not analysed.

View Independent Claims

Continued onto...

- Family 2. Modified hygromycin B resistance–conferring gene
- Family 3. Selectable marker for development of vectors and transformation systems in plants
Family 2. Modified hygromycin B resistance–confering gene

This family of patents is directed to modified DNA sequences of the hpt gene. The modified hpt gene is useful for cloning, isolating and characterizing promoters and also for constructing gene fusions that act as dominant selectable markers in appropriate host cells.

Differences to the wild-type sequence of the gene lie in the removal of the first, first and second, or first, second and third codons in the amino (N−) terminus of the HPT enzyme.

Plasmids bearing such truncated versions of the hpt gene are also part of the claimed invention. The plasmids also comprise non-native prokaryotic and eukaryotic transcriptional and translational activator sequences.

In addition, the Canadian and the United States patents claim the amino acid sequence of a modified HPT enzyme.

E. coli and Saccharomyces cerevisiae transformants with the vectors of the invention are also claimed in the Australian patent.

The disclosed modified sequence corresponds to the structural sequence of the hpt gene contained in the 1.45 kb restriction fragment of the pKC222 plasmid, except for the first N−terminal amino acids and the stop codon at the C−terminus, which are variable.

<table>
<thead>
<tr>
<th>Patents members of Family 2</th>
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<tr>
<td><strong>Country</strong></td>
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<tr>
<td>Australia</td>
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<td>Canada</td>
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<td>Hungary</td>
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<tr>
<td>Ireland</td>
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<tr>
<td>United States</td>
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</table>

* Patent term of the Canadian and United States patents is 17 years from the date of issuance.

** The European patent was converted to a national patent in Austria (AT), Belgium (BE), France (FR), Germany (DE), Italy (IT); Liechtenstein (LI); Luxemburg (LU); Netherlands (NL), Sweden (SE) and Switzerland (CH).

There are related patent applications pending in Denmark, Finland, Greece, Israel, and Japan.

To view or download the patents as PDF files, click on EP 135 291 B1 (2.7 kb) and US 4 960 704 (1.8 kb).

The modified hygromycin phosphotransferase sequence is inserted into a plasmid either alone or with a transcriptional and translational activator sequence. In the examples given, the inventors used bacterial sequences such as the coding sequence for the first 12 amino acids of the E. coli lacZ gene and eukaryotic sequences such as the yeast heat shock genes YG101 and YG100 and the phosphoglycerate kinase gene (PGK) as transcriptional and translational activator sequences.

The inventors state that other synthesized sequences encoding the same amino acids as those encoded by the disclosed sequence are within the scope of the invention.
Family 3. Selectable marker for development of vectors and transformation systems in plants

The patents of this family are directed to expression vectors for plant transformation containing chimeric genes that comprise a hpt gene. The hpt gene, also noted as an aphIV gene, serves as the basis for the selection of transformed plant cells.

The chimeric genes of the claimed invention contain from 5’ to 3’ direction:

a) a plant-expressible promoter sequence;

b) an aphIV gene which encodes a hygromycin phosphotransferase enzyme (US 5 668 298) or a functional portion of it (US 6 048 730†); and

c) a terminator signal sequence.

The US patent 5 668 298 claims a particular plasmid, pCEL40, which contains the promoter and the first 11 amino acids of the octopine synthase (OCS) gene of Agrobacterium Ti plasmid fused to an aphIV gene.

The independent claim of the European patent of this family is broader than in the United States patents. The components of the chimeric gene are not spelled out with the exception of “a coding region that confers hygromycin resistance on the plant cell”.

<table>
<thead>
<tr>
<th>Country</th>
<th>Granted Patent No.</th>
<th>Filing date</th>
<th>Issue date</th>
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<tr>
<td>United States</td>
<td>US 5 668 298†</td>
<td>June 7, 1995</td>
<td>September 16, 1997**</td>
</tr>
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<td></td>
<td>US 6 048 730†</td>
<td>September 19, 1990</td>
<td>April 11, 2000</td>
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* The European patent was converted to a national patent in Belgium (BE), France (FR), Germany (DE), Great Britain (GB), Italy (IT); Liechtenstein (LI); Luxembourg (LU); Netherlands (NL), Sweden (SE) and Switzerland (CH).

There is a related patent application pending in Denmark.

** Patent term of both United States patents is 17 years from the date of issuance.
† This United States patent was filed by and directly granted to Novartis.

To view or download the patents as PDF files, click on EP 186 425 B1 (1.3 kb), US 5 668 298 (1.0 kb) and US 6 048 730† (0.9 kb).
Thus, in Europe, any chimeric gene for plant transformation conferring resistance to hygromycin is possibly covered by the patented invention. Although, the United States patents are a bit more specific with respect to the comprising elements of the chimeric gene, the chimeric construct is described in such generic terms that it practically does not leave much freedom to operate for other constructs for plant transformation having an aphIV gene without infringing the patents.

View Independent Claims

As a conclusion...

The above group of patents pretty much covers the hygromycin phosphotransferase gene as a selectable marker for transformation of prokaryotes and eukaryotes. The broad coverage is not only with respect to the subject matter, but also to the geographical scope. Certainly, the inventions are protected in the main commercial jurisdictions: the United States, Europe, Japan, Australia and Canada. Essentially this group of patents owned by Eli Lilly and Novartis exclude others, almost completely, from this enabling technology tool. Hygromycin phosphotransferase gene is one of the most widely used selectable marker genes, mainly for monocot plants. This means, that most of the users of this gene, at least in the jurisdictions where the gene is protected, are likely to infringe the patents if the use has been unauthorised.

Back to...Overview

Next section... Dominant patents on antibiotic resistance genes.
### Claim 1
A recombinant DNA cloning vector comprising:

- a) a eukaryotic promoter;
- b) one or two different structural genes and associated control elements that convey resistance to either or both antibiotics hygromycin B and G418 when transformed into a host cell that is sensitive to either or both antibiotics for which resistance is conveyed, said host cell being susceptible to transformation, cell division and culture; and
- c) a prokaryotic replicon, said replicon being functional when said host cell is prokaryotic,

subject to the limitations

- that the one or two structural genes and associated control elements are adjacent to and, in a eukaryotic host cell, transcribed from the eukaryotic promoter,
- that a single gene and associated control sequence convey resistance to only either one of hygromycin B or G418, and
- that the gene conveying resistance to G418 does not code for the enzyme phosphotransferase.

### Claim 2
A recombinant DNA cloning vector comprising:

- a) a eukaryotic promoter;
- b) the 7.5 kb Bgl II restriction fragment of plasmid pKC203; and
- c) a prokaryotic replicon, said replicon being functional when said vector is transformed into a prokaryotic host cell,

subject to the limitations

- that said 7.5 kb Bgl II restriction fragment is adjacent to the eukaryotic promoter, and
- that any antibiotic resistance–confering gene contained within said 7.5 kb Bgl II restriction fragment is, in a eukaryotic host cell, transcribed from the eukaryotic promoter.

### Claim 20
A plasmid selected from the group consisting of plasmid pKC203, pKC222, pKC214, pKC215, pGD10, pGD11, pGD12, pGD13, pGD14 and pGD15¹.

¹ These *E. coli* plasmids contain either or both resistance genes for hygromycin and G418 in different orientations.
Claim 26
A restriction fragment selected from the group consisting of:

a) the 7.5 kb Bgl II restriction fragment of plasmid pKC203;
b) the 2.75 kb Bgl II/Sal I restriction fragment of plasmid pKC203;
c) the 1.5 kb Sac I/Bgl II restriction fragment of plasmid pKC222; and
d) the 1.65 kb EcoR I/Sal I restriction fragment of plasmid pKC222.

Claim 51
A transformed host cell selected from the group consisting of:

a) Mouse Ltk−/pKC214;
b) Mouse Ltk−/pKC215;
c) E. coli K12/pKC214;
d) E. coli K12/pKC215;
e) E. coli K12 BE827/pKC214;
f) E.coli K12 BE827/pKC215;
g) human/pKC214 cell; and
h) mammalian/ pKC214 cell.

Plasmids pKC214 and pKC215 have the 7.5 kb Bgl II restriction fragment containing resistance genes for both hygromycin and G418 in 5’ to 3’ and 3’ to 5’ direction, respectively.

Claim 59
A transformed host cell selected from the group consisting of:

a) E. coli K12 BE827/pKC222;
b) E. coli K12 BE827/pGD10;
c) E. coli K12 BE827/pGD11;
d) E. coli K12 BE827/pGD12;
e) E. coli K12 BE827/pGD13;
f) E. coli K12 BE827/pGD14;
g) E. coli K12 BE827/pGD15;
h) Mouse Ltk−/ pGD10;
i) Mouse Ltk−/ pGD11;
j) Mouse Ltk−/ pGD12;
k) Mouse Ltk−/ pGD13;
l) Mouse Ltk−/ pGD14; and
m) Mouse Ltk−/ pGD15.

Plasmid pKC222 contains the 2.5 kb Sal II/Bgl II restriction fragment with resistance genes for both hygromycin and G418.

Plasmids pGD10 and pGD11 contain each the 1.51 kb Bgl II/Sac I fragment with a hygromycin B resistance gene in different orientations.

Plasmids pGD12 and pGD13 contain each the 1.65 kb EcoR I/Sal I fragment with a G418 resistance gene in different orientations.

Plasmids pGD14 and pGD15 contain each the 2.75 kb Sal I/Bgl II fragment with resistance genes for both hygromycin and G418 in different orientations.
Claim 64
A plasmid selected from the group consisting of plasmid pSC701, pKC257, pKC259, pKC261, pKC275, pKC264, pLO378, pKC273, pLO314, pLO315, pLO316, pLO317, pLO318, pLO319, pLO320, and pLO321. The plasmid pSC701 contains the 7.5 Bgl II fragment with resistance genes for both hygromycin and G418. The plasmid pKC259 also confers resistance to both antibiotics. The remaining plasmids all convey resistance to hygromycin B only and are functional in both prokaryotic and eukaryotic host cells.

Claim 97
A eukaryotic host cell transformed with a vector selected from the group consisting of plasmids pLO378, pLO314, pLO315, pLO316, pLO317, pLO318, pLO319, pLO320, and pLO321. All these plasmids confer resistance to hygromycin B only.

Claim 98
A constructed hygromycin B phosphotransferase encoding DNA sequence, which comprises recombinant DNA, comprising the sequence:

\[
\text{[Sequence]}
\]

wherein A is deoxyadenyl, G is deoxyguanidyl, C is deoxycytisyl and T is thymidyl.

Claim 99
A constructed hygromycin B phosphotransferase encoding DNA sequence, which comprises recombinant DNA, comprising the sequence:

\[\text{[Sequence]}\]

wherein A is deoxyadenyl, G is deoxyguanidyl, C is deoxycytisyl and T is thymidyl.

1 DNA sequence corresponds to the coding sequence for the enzyme and associated control sequence.

Claim 100
A constructed DNA sequence that comprises recombinant DNA that encodes a polypeptide comprising the amino acid sequence

\[\text{[polypeptide sequence]}\]

wherein MET is methionine, LYS is lysine, PRO is proline, GLU is glutamic acid, LEU is leucine, THR is threonine, ALA is alanine, SER is serine, VAL is valine, PHE is phenylalanine, ILE is isoleucine, GLY is glycine, ASP is aspartic acid, GLN is glutamine, ARG is arginine, CYS is cysteine, TRP is tryptophan, ASN is asparagine, HIS is histidine and TYR is tyrosine.

10 Sequence corresponds to the 341 amino acid residues of hygromycin B phosphotransferase.
Claim 1
A recombinant DNA cloning vector comprising:

a) a eukaryotic promoter;
b) one or two different structural genes and associated control sequence that convey resistance to either or both antibiotics hygromycin B and G418 when transformed into a host cell that is sensitive to either or both antibiotics for which resistance is conveyed, said host cell being susceptible to transformation, cell division, and culture; and
c) a prokaryotic replicon, said replicon being functional when said host cell is prokaryotic,

subjected to the limitations

- that the one or two structural genes and associated control sequence are adjacent to and, in a eukaryotic host cell, transcribed from the eukaryotic promoter,
- that a single gene and associated control sequence conveys resistance to either but not both hygromycin B and G418, and
- that the gene conveying resistance to G418 does not code for the enzyme phosphotransferase.

Claim 16
A transformed host cell which comprises a recombinant DNA cloning vector comprising:

a) a eukaryotic promoter;
b) one or two different structural genes and associated control sequence that convey resistance to either or both antibiotics hygromycin B and G418 when transformed into a host cell that is sensitive to either or both antibiotics for which resistance is conveyed, said host cell being susceptible to transformation, cell division, and culture; and
c) a prokaryotic replicon, said replicon being functional when said host cell is prokaryotic,

subjected to the limitations

- that the one or two structural genes and associated control sequence are adjacent to and, in a eukaryotic host cell, transcribed from the eukaryotic promoter,
- that a single gene and associated control sequence conveys resistance to either but not both hygromycin B and G418, and
- that the gene conveying resistance to G418 does not code for the enzyme phosphotransferase.

Claim 33
A restriction fragment selected from the group consisting of:

a) the 7.5 kb Bgl II restriction fragment of plasmid pKC203;
b) the 2.75 kb Bgl II/Sal I restriction fragment of plasmid pKC203;
c) the 1.51 kb Sac I/Bgl II restriction fragment of plasmid pKC222; and
d) the 1.65 kb EcoR I/Sal I restriction fragment of plasmid pKC222.
Claim 34
A plasmid selected from the group consisting of plasmid pKC203, pKC222, pKC257, pKC259, pKC261, pKC264, pKC275, and pSC701.

11 The plasmids pSC701 contains the 7.5 Bgl II fragment with resistance genes for both hygromycin and G418. The plasmid pKC259 also confers resistance to both antibiotics. The remaining plasmids all convey resistance to hygromycin B only. From them, plasmids pKC257, pKC259, pKC261 are functional in prokaryotes, and plasmids pKC264 and pKC275 are functional in eukaryotic host cells.

Claim 35
A method for producing post translationally modified polypeptide, which comprises:

a) transforming a eukaryotic cell with a recombinant DNA cloning vector comprising:

i. a eukaryotic promoter;

ii. one or two different structural genes and associated control sequence that convey resistance to either or both antibiotics hygromycin B and G418 when transformed into a host cell that is sensitive to either or both antibiotics for which resistance is conveyed, said host cell being susceptible to transformation, cell division, and culture; and

iii. a prokaryotic replicon, said replicon being functional when said host cell is prokaryotic,

subjected to the limitations

♦ that the one or two structural genes and associated control sequence are adjacent to and, in a eukaryotic host cell, transcribed from the eukaryotic promoter,

♦ that a single gene and associated control sequence conveys resistance to either but not both hygromycin B and G418, and

♦ that the gene conveying resistance to G418 does not code for the enzyme phosphotransferase, and

b) culturing the transformed cell.

Actual granted independent claims

EP 68740 B1

Claim 1
Plasmid pKC222 which contains the 2.75 kb Sal I/Bgl II restriction fragment of plasmid pKC203 as obtainable from E. coli JR225 ATCC 31912 ligated to the Sal I/Bgl II restriction fragment of plasmid pKC7, and which confers resistance to antibiotics ampicillin, hygromycin B and G418 when transformed into an E. coli cell.
Claim 2
A recombinant DNA cloning vector that comprises a eukaryotic promoter, the plasmid pBR322 replicon, and

a) the 7.5 kb Bgl II restriction fragment of plasmid pKC203 that conveys resistance to antibiotics hygromycin B and G418; or
b) the 2.75 kb Bgl II/Sal I restriction fragment of plasmid pKC203 or plasmid pKC222 that conveys resistance to antibiotics hygromycin B and G418; or
c) the 1.51 kb Sac I/Bgl II restriction fragment of plasmid pKC222 that conveys resistance to antibiotic hygromycin B; or

d) the 1.65 kb Eco RI/Sal I restriction fragment of plasmid pKC222 that conveys resistance to antibiotic G418; or
e) a DNA sequence derived from the said 7.5 kb restriction fragment that conveys resistance to either or both antibiotics hygromycin B and G418;

the resistance to antibiotics hygromycin B and/or G418 being conveyed when the vector is transformed into a host cell that is sensitive to one or both of said antibiotics, the host cell being susceptible to transformation, cell division, and culture;

subject to the limitations

- that the promoter is functional in mouse or yeast cells,
- that the restriction fragments conveying antibiotic resistance are adjacent to and, in a eukaryotic host cell, transcribed from the promoter, and
- that the DNA sequences conveying resistance to antibiotic G418 do not code for the enzyme phosphotransferase.

Claim 22
Plasmid pSC701 obtained by self-ligation of the 7.3 kb Bgl II restriction fragment of plasmid pKC203 (ATCC 31912) and having the restriction map shown in Figure 6.

Claim 23
Plasmid pKC257 obtained by incubating plasmid pSC701 with Hae II restriction enzyme, self-ligating the resulting mixture of restriction fragments, transforming an E. coli K12 strain with the ligated mixture, and screening the transformants for hygromycin B resistance; and having the molecular weight and restriction map shown in Figure 7.

Claim 24
Plasmid pKC259 obtained by incubating plasmid pSC701 with Hae II restriction enzyme, self-ligating the resulting mixture of restriction fragments, transforming an E. coli K12 strain with the ligated mixture, and screening the transformants for ampicillin and hygromycin B resistance; and having the molecular weight and restriction map shown in Figure 7.

Claim 25
Plasmid pKC261 obtained by self-ligation of the 3.2 kb Sau3A I restriction fragment of plasmid pKC257 and having the restriction map shown in Figure 7.
| Claim 26 | Plasmid pKC275 obtained by ligating the 396 base plac containing Hae II restriction fragment of plasmid pUR222 as obtainable from *E. coli* K12 BE1166 NRRL B−15023 and a mixture of Hae II restriction fragments of plasmid pKC261, transforming and *E. coli* K12 strain with the ligated mixture, and selecting transformants containing only a 3.6 kb plasmid; and having the restriction map shown in Figure 8. |
| Claim 27 | Plasmid pKC264 obtained by ligating the 2 µ EcoR I restriction fragment of plasmid YEp24 as obtainable *E. coli* K12 BE1139 NRRL B−15022 and a mixture of EcoR I restriction fragments of plasmid pKC259, transforming an *E. coli* K12 strain with the ligated mixture, and selecting transformants containing an 7.2 kb hygromycin B, apramycin and G418 resistance−conferring plasmid; and having the restriction map shown in Figure 8. |
| Claim 28 | The 7.5 kb Bgl II restriction fragment of plasmid pKC203. |
| Claim 29 | The 2.75 kb Sal I/Bgl II restriction fragment of plasmid pKC203. |
| Claim 30 | The 1.51 kb Sac I/Bgl II restriction fragment of plasmid pKC222. |
| Claim 31 | The 1.65 kb EcoR I/Sal I restriction fragment of plasmid pKC222. |
### Claim 1
A recombinant DNA plasmid which comprises:

a) a eukaryotic promoter;

b) one or two different structural genes and associated control sequence that convey resistance to either or both of antibiotics hygromycin B and G418 when transformed into a host cell that is sensitive to either or both antibiotics for which resistance is conveyed, said host cell being susceptible to transformation, cell division, and culture; and

c) a prokaryotic replicon, said replicon being functional when said host cell is prokaryotic,

subject to the limitations

- that the one or two structural genes and associated control sequence are adjacent to and, in a eukaryotic host cell, transcribed from the eukaryotic promoter,
- that a single gene and associated control sequence conveys resistance to either but not both hygromycin B and G418, and
- that the gene conveying resistance to G418 does not code for the enzyme phosphotransferase.

### Claim 19
A recombinant DNA cloning vector substantially as described with reference to Examples 3, 7, 10, 16, 19, 22, 30, 33, 35, 37, 39, and 41.

The examples refer to:

- Ex. 3, 7 and 10: construction of plasmids pKC214, pKC215, pGD1, pGD2, pGD3 and pGD4 with the 7.5 kb Blg II fragment having both hygromycin and G418 resistance genes in different orientations.
- Ex. 16: construction of plasmids pGD10 and pGD11 with the 1.51 kb Sac I/Blg II fragment having a hygromycin resistance gene in different orientations.
- Ex. 19: construction of plasmids pGD12 and pGD13 with the 1.65 kb EcoR I/Sal I fragment having a G418 resistance gene in different orientations.
- Ex. 22: construction of plasmids pGD14 and pGD15 with the 2.5 kb Sal I/Bgl II fragment having a G418 resistance gene in different orientations.
- Ex. 30: construction of plasmids pLO314 and pLO315 with both hygromycin and G418 resistance genes in different orientations functional in prokaryotes and eukaryotes.
- Ex. 39 and 41: construction of plasmids pKC273 and pLO378 with both hygromycin and G418 resistance genes and functional in prokaryotes.

### Claim 20
A transformed host cell substantially as described with reference to Examples 3 to 5, 7 to 12, 16 to 24, and 31 to 42.

The transformed host cells referred to in the examples are:

- Ex. 3 to 5: Mouse cells.
- Ex. 7 to 12: Mouse, yeast and *E. coli* cells.
- Ex. 16 to 24: Mouse and *E. coli* cells.
- Ex. 31 to 42: Mouse, *Saccharomyces* and *E. coli*
Actual granted independent claims

<table>
<thead>
<tr>
<th>Claim 1</th>
<th>A DNA sequence having one or two different structural genes and associated control sequence which conveys resistance to either or both of antibiotics hygromycin B and G418 and which is:</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>the 7.5 kb Bgl II restriction fragment,</td>
</tr>
<tr>
<td>b)</td>
<td>the 2.75 kb Bgl II/Sal I restriction fragment,</td>
</tr>
<tr>
<td>c)</td>
<td>the 1.51 kb Sac I/Bgl II restriction fragment,</td>
</tr>
<tr>
<td>d)</td>
<td>the 1.65 kb Eco RI/Sal I restriction fragment of plasmid pKC203.</td>
</tr>
</tbody>
</table>

| Claim 5 | A DNA sequence having one or two different structural genes and associated control sequence which conveys resistance to either or both of antibiotics hygromycin B and G418 substantially as hereinbefore described with particular reference to Examples 2 and 4 to 6. |

14 The examples refer to:
Ex. 2: the isolation of the 7.5 kb fragment having both antibiotic resistance genes from plasmid pKC203; and
Ex. 4 to 6: the isolation of 1.51 Kb and 1.65 kb fragments from the plasmid pKC222 containing the gene for hygromycin resistance and for G418 resistance respectively.

| Claim 6 | A recombinant DNA cloning vector substantially as described with particular reference to Examples 1, 4, and 7 to 11. |

15 The examples refer to:
Ex. 1: the construction of plasmid pKC203 having the 7.5 kb fragment with both antibiotic resistance genes; Ex. 4: the construction of plasmid pKC222 having the 2.5 kb fragment with both antibiotic resistance genes; Ex. 7: the construction of plasmid pSC701 having the 7.5 kb fragment with both antibiotic resistance genes; Ex. 8: the construction of plasmid pKC257 with a fragment containing a hygromycin resistance gene and the plasmid pKC259 with a fragment containing resistance genes for both hygromycin and G418; and Ex. 9 to 11: the construction of plasmid pKC261, pKC264 and pKC275 with a fragment containing a hygromycin resistance gene.

| Claim 7 | A transformed host cell substantially as hereinbefore described with particular reference to Examples 1, 4, and 7 to 11. |

16 All examples refer to *E. coli* transformation.
Claim 1
A DNA sequence which encodes the amino acid sequence:
[sequence]

wherein MET is methionine, LYS is lysine, PRO is proline, GLU is glutamic acid, LEU is leucine, THR is threonine,ALA is alanine,SER is serine,VAL is valine,PHE is phenyalanine,ILE is isoleucine,GLY is glycine, ASP is aspartic acid, GLN is glutamine, ARG is arginine, CYS is cysteine, TRP is tryptophan, ASN is asparagine, HIS is histidine and TYR is tyrosine, and

R is a dideoxyribonucleotide triplet that encodes lysine and z=0 to 2;
subject to the limitation that said DNA encodes a hygromycin B phosphotransferase that is not encoded by plasmid pKC203.

Claim 30
The plasmid pIT207¹.

¹ This plasmid contains the yeast heat shock gene (YG101) and the truncated hpt gene.

Claim 46
A plasmid selected from the group consisting of plasmids pIT141, pIT143, pIT212, pIT213, pIT215, pIT217, and pIT219².

² These plasmids contain either the transcriptional and activator sequences of the phosphoglycerate kinase gene (PGK) or of the yeast heat shock gene (YG101) fused to the truncated hpt gene.
### Claim 1
A DNA encoding the last 338, 339 or 349 amino acids of hygromycin B phosphotransferase.

### Claim 15
Plasmid pIT123 which is shown in Figure 1 and constructed by the steps of:

a) ligating the 2.75 kb Sal I/Bgl II fragment of plasmid pKC203 (ATCC Deposit No. 31912) and the 4.1 kb Sal I/Bgl II fragment of plasmid pKC7 (ATCC Deposit No. 37084) to form plasmid pKC222;
b) providing the 325 bp Hph I−Pst I fragment of plasmid pKC222 with a BamH I linker and an EcoR I linker;
c) ligating the linker provided fragment of step (b) with the EcoR I−BamH I digest of known plasmid pBR322 to provide plasmid pIT122; and
d) ligating the 1.45 kb EcoR I fragment of plasmid pKC222 and the EcoR I digest of plasmid pIT122.

### Claim 1
A process for preparing a plasmid comprising a DNA sequence encoding the last 338 amino acids of hygromycin B phosphotransferase, either alone or in translational reading phase with a transcriptional and translational activator sequence−containing gene or portion of a gene, which comprises the steps of:

a) ligating the 2.75 kb Sal I/Bgl II fragment of plasmid pKC203 (ATCC Deposit No. 31912) and the 4.1 kb Sal I/Bgl II fragment of plasmid pKC7 (ATCC Deposit No. 37084) to form plasmid pKC222;
b) providing the 325 bp Hph I−Pst I fragment of plasmid pKC222 with a BamH I linker and an EcoR I linker;
c) ligating the linker provided fragment of step (b) with the EcoR I−BamH I digest of known plasmid pBR322 to provide plasmid pIT122; and
d) ligating the 1.45 kb EcoR I fragment of plasmid pKC222 and the EcoR I digest of plasmid pIT122, to obtain the plasmid pIT123 shown in Figure 1.

### Claim 9
The process for preparing plasmid pIT207, shown in Figure 3, which comprises ligating the 750 bp BamH I−Bgl II fragment of plasmid pIT118 (NRRL deposit No. B−15441) into BamH I−digested plasmid pMC1587 (NRRL deposit No. B−15442).

### Claim 11
The process for preparing plasmid pIT143 which comprises:

a) digesting the 958 bp Cla I−Hinc II fragment of plasmid pIT141 (NRRL deposit No. B−15602) with the restriction enzyme Mbo II;
b) removing the resultant extensions attaching BamH I linkers, and
c) ligating the linker−containing fragment into BamH I−digested plasmid pUC8.
**Claim 1**
A DNA encoding all the amino acids of hygromycin B phosphotransferase except with reference to the N-terminus of the naturally occurring hygromycin B phosphotransferase molecule, the first, the first and second, or the first, second and third amino acids, either alone or in translational phase with a gene or portion of a gene,

said gene or portion thereof containing a transcriptional and translational activator sequence, subject to the limitation that said DNA is not associated with the transcriptional and translational activator sequence of plasmid pKC203 as obtainable from *E. coli* JR225 deposited with ATCC under accession number ATCC 31912,

said DNA encoding the following amino acid sequence:

```
[sequence]
```

wherein m and n=0 or 1, subject to the limitation that when n=0, then m=0 and when m=1, then n=1, and wherein MET is methionine, LYS is lysine, PRO is proline, GLU is glutamic acid, LEU is leucine, THR is threonine, ALA is alanine, SER is serine, VAL is valine, PHE is phenyalanine, ILE is isoleucine, GLY is glycine, ASP is aspartic acid, GLN is glutamine, ARG is arginine, CYS is cysteine, TRP is tryptophan, ASN is asparagine, HIS is histidine and TYR is tyrosine.

**Claim 34**
The plasmid pIT207.

*The same as claim 30 of the United States patent.

**Claim 35**
A plasmid selected from the group consisting of plasmids pIT141, pIT143, pIT212, pIT213, pIT215, pIT217, and pIT219.

**The same as claim 46 of the United States patent.

**Claim 1**
A novel DNA encoding the last 338, 339 or 340 amino acids of hygromycin B phosphotransferase, either alone or in translational reading phase with a transcriptional and translational activator sequence-containing gene or portion of a gene.

**Claim 22**
Plasmid pIT141, pIT143 or pIT207 as hereinbefore defined.

**Claim 24**
A novel DNA encoding the last 338, 339 or 349 amino acids of hygromycin B phosphotransferase substantially as hereinbefore described with particular reference to Examples 2 to 4, 6 and 7, and 9 to 13.

**Claim 25**
A recombinant DNA cloning vector substantially as hereinbefore described with particular reference to Examples 2 to 4, 6 and 7, and 9 to 13.
Claim 26
A transformant comprising a recombinant DNA cloning vector substantially as hereinbefore described with particular reference to Examples 2 to 7, and Table 3.
Selectable marker for development of vectors and transformation systems in plants.

Patents granted to Eli Lilly Co.

Actual granted independent claims

This patent was assigned to Novartis

### US 5 668 298

<table>
<thead>
<tr>
<th>Claim 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A chimeric gene functional in a plant cell, which chimeric gene comprises:</td>
</tr>
<tr>
<td>a) at a position 5' to coding region (c), a plant–expressible promoter sequence;</td>
</tr>
<tr>
<td>b) at a position 3' to coding region (c), a terminator signal sequence; and</td>
</tr>
<tr>
<td>c) a coding region of an aphIV gene, which coding region:</td>
</tr>
</tbody>
</table>

i. encodes a functional hygromycin phosphotransferase enzyme; and  
ii. is positioned between such plant–expressible promoter sequence (a) and such terminator signal sequence (b) so as to be expressible,  
wherein expression of such coding region in a plant cell confers resistance to hygromycin B on such plant cell and  
wherein such resistance to hygromycin B is capable of providing a basis for selection of such plant cell.

<table>
<thead>
<tr>
<th>Claim 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid pCEL40 shown in Fig. 2 and derivable by ligating the Bgl II digest of plasmid pCEL30, to the about 1.3 kb BamH I–Bgl II fragment of plasmid pOW20, such plasmids pCEL30 and pOW20 being obtainable from NRRL B–15915 and NRRL B–15838, respectively.</td>
</tr>
</tbody>
</table>

This patent was applied by and granted to Novartis

### US 6 048 730

<table>
<thead>
<tr>
<th>Claim 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A chimeric gene functional in a plant cell, which chimeric gene comprises:</td>
</tr>
<tr>
<td>a) at a position 5' to coding region (c), a plant–expressible promoter sequence;</td>
</tr>
<tr>
<td>b) at a position 3' to coding region (c), a terminator signal sequence; and</td>
</tr>
<tr>
<td>c) a coding region of an aphIV gene, which coding region:</td>
</tr>
</tbody>
</table>

1. encodes a functional hygromycin phosphotransferase enzyme or functional portion thereof; and  
2. is positioned between such plant–expressible promoter sequence (a) and such terminator signal sequence (b) so as to be expressible,  
wherein expression of such coding region in a plant cell confers resistance to hygromycin B on such plant cell and  
wherein such resistance to hygromycin B is capable of providing a basis for selection of such plant cell.
<table>
<thead>
<tr>
<th><strong>Claim 1</strong></th>
<th>A chimeric gene functional in a plant cell that comprises a coding region that confers hygromycin resistance on the cell, wherein the hygromycin resistance is capable of providing the basis for selection of the cell.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Claim 21</strong></td>
<td>Plasmid pCEL30 shown in Figure 1 and obtainable from NRRL B−15915.</td>
</tr>
<tr>
<td><strong>Claim 22</strong></td>
<td>Plasmid pCEL40 shown in Figure 2 and derivable by ligating the Bgl II digest of plasmid pCEL30, which plasmid pCEL30 is obtainable from NRRL B−15915, and the 1.3 kb BamHI–Bgl II fragment of plasmid pOW20, which plasmid pOW20 is obtainable from NRRL B15838.</td>
</tr>
</tbody>
</table>
Dominant patents on antibiotic resistance genes

Comparison between patents on antibiotic resistance genes in general, nptII and hygromycin

Are there dominant patents in this field?

One can say, yes, in the United States there are dominant patents on the use of antibiotic resistance genes for the selection of transformed plants. That means, it is likely that any other patented invention on this subject matter or any user of antibiotic resistance genes would not have freedom to operate unless permission is granted for the use of the dominant inventions.

The dominant patents on this field are owned by Monsanto and cover a chimeric gene having any antibiotic resistance gene (see analysis in Antibiotic resistance genes in general). The patents differ with respect to the type of promoter used to control the antibiotic resistance gene as follows:

<table>
<thead>
<tr>
<th>Patent Number</th>
<th>Promoter Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>US 6 174 724</td>
<td>any promoter naturally expressed in plants</td>
</tr>
<tr>
<td>US 6 255 560</td>
<td>CaMV 35S and 19S</td>
</tr>
<tr>
<td>US 5 034 322</td>
<td>opine synthase and rbcS</td>
</tr>
</tbody>
</table>

Of these, the first one, US 6 174 724, is the broadest. As discussed in Antibiotic resistance genes in general any promoter that expresses naturally in plants may include any promoter derived from any plant gene and also from genes whose expression only occurs in a plant cell. As long as any of the sorts of promoters claimed in these patents is used in a chimeric gene with any antibiotic resistance gene, the construct will be likely covered by the claims.

It means that nptII and hpt genes and even other antibiotic resistance genes not analysed in this paper, such as bleomycin resistance gene conferring resistance to bleomycin and phleomycin and genes conferring resistance to gentamycin and streptomycin, could be covered by the claims of this patent, if they are part of a chimeric gene having the patented elements.

What are the limits?

The limits to the breadth of the patents are mainly

- In the promoters

  If you think of any other promoter that does not fall within any of the claimed groups, a devised chimeric gene might be outside of the claims.
In the country where the patents are issued
These broad patents are only in force in the United States. Patents with such broad scope have not been granted in any other country. Therefore, Monsanto can be only enforce its patent rights over the inventions in the United States.

You may ask whether the use of these patented chimeric genes in a non–plant organism (e.g., animal cell) would be non–infringing? Remember though that patent rights allow the owner to exclude others from "making, using, selling, or offering to sell". Because these patents are protecting products, not processes, the sort of use of a protected product is irrelevant. As long as you have made or used (or sell or offer to sell) a product that comprises the elements of the claimed chimeric genes, you would be infringing.

In what way are the patents on nptII and hpt genes dominated by this trio of patents?

This question is limited to the United States as the dominant patents are only granted and in force in this country.

• nptII patents

Monsanto is also the owner of patents on the use of nptII as antibiotic resistance gene for plant transformation (see IP aspects of the npt gene). Therefore, Monsanto is in an advantageous position by having the patent rights on any antibiotic resistance gene as well as on a gene encoding a neomycin phosphotransferase enzyme. In the latter patent, it means that apart from the nptII gene, other genes coding for a neomycin phosphotransferase, i.e. npt, could be encompassed by the patent claims.

The other United States patents discussed in the section IP aspects of nptII gene are directed to constructs that may not fall within the limits of the Monsanto's patents on any antibiotic resistance gene. The bifunctional marker by the National Research Council of Canada has the nptII gene linked to a gus gene and is not limited to particular organisms. So, in principle, the fusion gene appears to be outside the scope of the Monsanto patents. However, if the construct having the fusion gene comprised the elements claimed in the ‘trio’ of patents, then, despite having in addition the gus gene, the construct might infringe the protected Monsanto's chimeric genes. The transition word "comprising" used in the Monsanto's claims means that the claimed chimeric genes contain all the elements listed but can also include additional elements. Therefore, having a chimeric gene with all the claimed elements plus the gus gene does not avoid infringement.

The aminoglycoside phosphotransferase gene claimed by Cetus Corporation might fall under Monsanto's patents, if the expression vector containing the modified truncated aphI gene, which is an antibiotic resistance gene, encompassed any of the promoters claimed by Monsanto controlling the gene and a poly(A) signal. Furthermore, such expression vector might only be infringing if it was capable of being used for plants. Other eukaryotic organisms are not covered by the Monsanto's claims.

• hpt patents

Despite the solid patent portfolio of Eli Lilly's and Novartis’ on cloning vectors containing the hpt gene (see IP aspects of the hpt gene), the claimed constructs for plant transformation, in particular, appear to be encompassed by Monsanto's protected chimeric genes. Once more, this situation is only applicable to the United States patents, which are assigned to Novartis.

The United States hpt patents that are members of families 1 and 2 encompass more than plants, including prokaryotic and eukaryotic organisms. The recombinant DNA cloning vectors of the United States patent of family
Antibiotic resistance genes and its use in genetic transformation, especially in plants

No. 1 would have to contain a eukaryotic promoter falling within one of the types of promoters claimed by the Monsanto ‘trio’ of patents to be encompassed by the Monsanto patents. The claimed plasmids of the United States patent of the family No. 2 contain promoters that are either derived from yeast genes or from eukaryotic genes which are not naturally expressed in plants. Therefore, these plasmids are not likely engulfed by the Monsanto's patents.

In contrast, the chimeric genes claimed in the two United States Novartis patents of family No. 3 are more likely to be dominated by the Monsanto patents on antibiotic resistance genes in general. The Novartis chimeric genes confer plants resistance to the hygromycin antibiotic.

These chimeric constructs comprise a plant–expressible promoter and a terminal signal sequence apart from the hygromycin resistance coding region. These are akin to the constructs claimed by Monsanto, but if the promoter and the terminal signal sequences are not the same as the ones claimed by Monsanto, the Novartis' chimeric genes could not be encompassed by the Monsanto's constructs.
Synopsis

Antibiotic resistance genes white paper

Antibiotic resistance genes are widely used as selectable markers because they are highly efficient, economical and straightforward. Therefore, they are considered a very valuable tool at experimental and commercial research levels.

As with many enabling technologies, these are proprietary technologies in the hands of few entities. The scope of patent protection ranges from the very broadly claimed use of any antibiotic resistance gene in plant transformation to the more restrictive use of particular antibiotic resistance systems in conjunction with particular promoters and selective agents.

The present paper analyses the extent of patent protection on

- the use of any antibiotic resistance gene, mainly for plant transformation
- the most commonly used antibiotic resistance marker genes: neomycin phosphotransferase (npt) and hygromycin phosphotransferase (hpt).

A summary of the information contained within this paper is presented in the following table. A total of six different entities holding 21 patents are part of the analysis. The table provides a listing of the entities having patents on this field, the patent document number and a brief description of the invention claimed in the analysed patents. For detailed information on the patents within a particular section, follow the relevant link under the “Entity” column.

<table>
<thead>
<tr>
<th>Sections</th>
<th>Antibiotic resistance genes in general</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entity</td>
<td>Monsanto Co.</td>
</tr>
<tr>
<td></td>
<td>US 6 174 724</td>
</tr>
<tr>
<td></td>
<td>US 6 255 560</td>
</tr>
<tr>
<td>Claimed invention</td>
<td>A chimeric construct comprising any antibiotic resistance gene under control of a promoter that works in plants.</td>
</tr>
<tr>
<td></td>
<td>Promoter controlling the antibiotic resistance gene derived from:</td>
</tr>
<tr>
<td></td>
<td>US 5 034 322: A. tumefaciens opine synthase gene &amp; rbcS gene</td>
</tr>
<tr>
<td></td>
<td>US 6 174 724: a gene &quot;naturally expressed&quot; in plants</td>
</tr>
<tr>
<td></td>
<td>US 6 255 560: 35S CaMV and 19S CaMV.</td>
</tr>
</tbody>
</table>
Antibiotic resistance genes and its use in genetic transformation, especially in plants

<table>
<thead>
<tr>
<th>Neomycin phosphotransferase (<em>npt</em>) gene</th>
</tr>
</thead>
</table>
| **Monsanto Co.** | US 5 034 322  
US 6 174 724  
EP 131 623 B2  |

- Chimeric constructs for plant transformation containing a neomycin phosphotransferase-encoding gene, which confers resistance to aminoglycoside antibiotics.
- Promoter controlling the antibiotic resistance gene derived from:
  - **US 5 034 322**: *A. tumefaciens* opine synthase gene & *rbcS* gene
  - **US 6 174 724**: a gene “naturally expressed” in plants

| **Japan Tobacco** | EP 927 765 A1* |

- Use of the *nptII* gene in combination with the antibiotic *paromomycin* for the selection of transformed rice cells.

| **National Research Council of Canada** | US 5 639 663  
EP 583 258 B1  |

- A fused gene comprising a reporter marker gene (*gusA* gene) and an antibiotic resistance gene (*nptII* gene).

| **Cetus Corporation** | US 4 784 949  
CA 1337716 A1  |

- A DNA sequence of a modified truncated *aph-I* gene. The modified gene is particularly effective against G418 and neomycin antibiotics.

<table>
<thead>
<tr>
<th>Hygromycin phosphotransferase (<em>hpt</em>) gene</th>
</tr>
</thead>
</table>
| **Eli Lilly Co./Novartis** | US 4 727 028  
US 4 960 704  
US 5 668 298  
US 6 048 730  
EP 68740 B1  
EP 135 291 B1  
EP 186 425 B1  
CA 1195626 A1  
CA 1278540 A1  
AU 555 574 B2  
AU 565 625 B2  
AU 582 653 B2  |

- Recombinant DNA cloning vectors that confer resistance to hygromycin and G418 antibiotics in both prokaryotic and eukaryotic cells.
- A modified truncated DNA sequence of the *hpt* gene.
- Vectors for plant transformation containing chimeric genes that comprise a *hpt* gene.

* This European patent document corresponds to a patent application. The rest of the patent documents that appear in the table are granted patents. The latest status of the patent documents was checked in August 2002.