Chapter 4
Genetic Engineering of Living Cells and Organisms

TERJE TRAAVIK,[1, 3] KAARE M. NIELSEN[1, 2] AND DAVID QUIST[1]
[1]THE NORWEGIAN INSTITUTE OF GENE ECOLOGY (GENØK), TROMSØ, NORWAY
[4]DEPARTMENT OF PHARMACY, UNIVERSITY OF TROMSØ, NORWAY
[3]DEPARTMENT OF MICROBIOLOGY AND VIROLOGY, UNIVERSITY OF TROMSØ, NORWAY

Genetic engineering (GE) by *transgenesis* has three main application areas: medicine, agriculture and bioremediation of the environment. In all three areas, transgenic crop plants, livestock, microorganisms and viruses are used. In the future we will increasingly be confronted also with transgenic trees, insects, fish species, and viruses. A development towards multi-transgenic, 'stacked', constructs is anticipated. Finally, multi-transgenic organisms based on nanobiotechnology, RNAi technology and 'synthetic biology', used separately or in combinations, may become realities.

This chapter provides a broad overview of strategies and techniques that are being used, or will be used in the near future, to produce transgenic organisms. This chapter is structured according to the following outline:

1. The processes involved in making a genetically modified organism
- 1.1 General strategies for making a GMO
- 1.2 Sources of transgenes
- 1.3 Vector construction for gene transfer into higher organisms: General aspects

2. Insertion of genes into plants

3. Insertion of genes into animals

4. Insertion of genes into microorganisms

5. Location of the inserted genes

6. Future prospects of gene transfer methodologies

1. The processes involved in making a genetically modified organism

1.1 General strategies for making a GMO
A number of strategies for physical transfer of DNA into cells are available. Some of these are generally applicable, while others are only feasible for cells from specific sources. The strategies and approaches used are often collectively termed *recombinant DNA technology.* The term comprises an arsenal of laboratory methods used to identify and isolate a DNA fragment from one organism, insert it into a vector and transfer the vector-insert combination into a host cell. The vector is often a bacterial *plasmid*. The process would not be possible without «biological scissors», i.e. *enzymes (restriction endonucleases)* that reproducibly cleave DNA molecules into fragments of defined sizes. Furthermore, the process requires 'biological glue', i.e. enzymes called *ligases*, to join the insert and vector together. A generic gene cloning process may be divided into some general steps as illustrated in Figure 4.1.

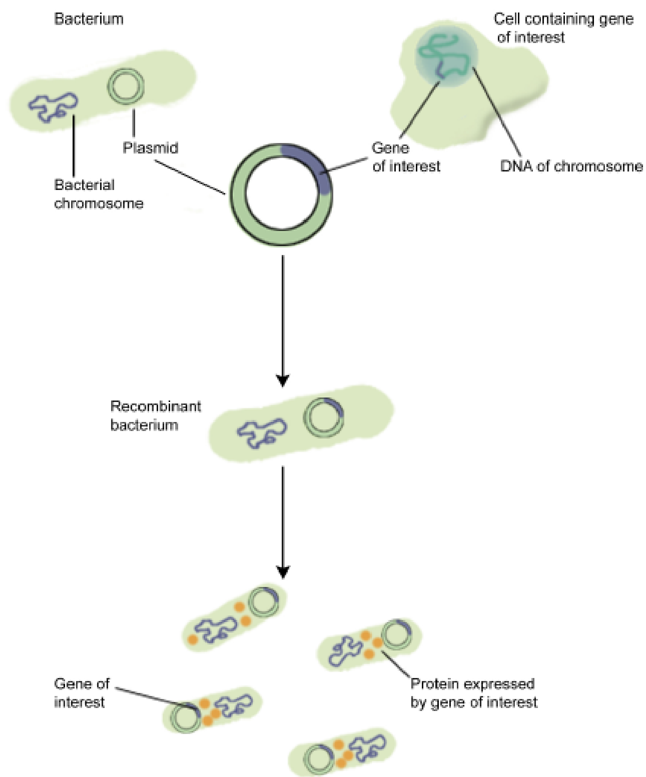

Figure 4.1. Simplified outline of DNA cloning and gene expression in bacteria.

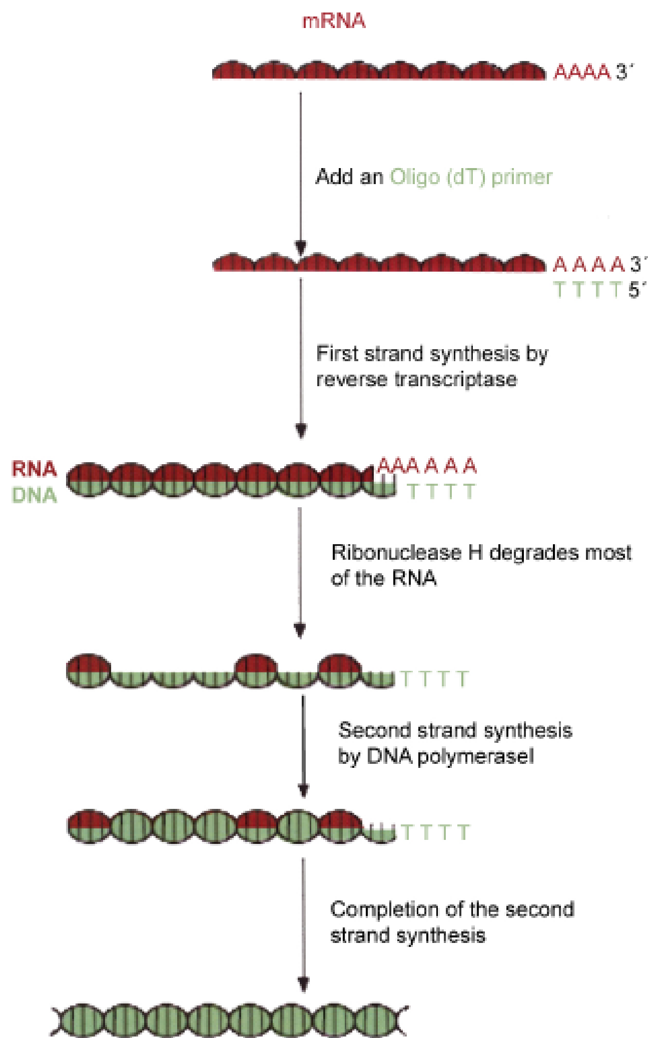

Figure 4.2. Outline of a reverse transcription process.

It is important to acknowledge that, due to the presence of introns, many eukaryotic genes are too large for direct cloning into bacterial vectors. Hence, cloning of eukaryotic genes is commonly initiated by isolation of the corresponding already processed mRNAs. The mRNA is then reversely transcribed into a double-stranded *cDNA* (complementary DNA). This is achieved by a *reverse transcriptase enzyme* making the first strand and a *DNA polymerase* making the second strand. The process is outlined in Fig. 4.2. The completed cDNA contains the open reading frame necessary for expressing the protein originally encoded in the genomic DNA (with the introns).

Table 4.1 The general steps involved in a generic gene cloning process

Gene isolation and excision	The DNA or mRNA is isolated from an organism that contains the target gene (e.g. a Bt toxin (*cry*) gene from *Bacillus thuringiensis*). In the case of DNA it is cut with a restriction endonuclease.
Vector preparation	The chosen DNA cloning vector is cut with the same restriction endonuclease.
Ligation	The two DNA samples are pasted together by a *DNA ligase* to produce *recombined molecules*.
Transformation with the vector	*E. coli* cells are transformed with the combined DNA molecules from the ligase reaction to produce cells that carry the target gene-vector recombinant molecules. The vector contains a DNA sequence, *origin of replication (ori)*, that enables it to be replicated in *E. coli*, and hence the recombinant molecules may be replicated into a high number of copies. Uptake of DNA in *E. coli* may be facilitated by a number of procedures, e.g. $CaCl_2$ – heat shock treatment or electroporation.
Marker and target gene expression	*E. coli* cells containing the vector, and hence the target gene, are selected on the basis of an *antibiotic resistance (AR) gene* which is an integral part of the vector. When the corresponding antibiotic (e.g. ampicillin, kanamycin or neomycin) is added, only cells containing the recombinant vector molecules will survive the treatment.

1.2 Sources of transgenes

Any kind of organism may be a source of useful transgenes. Transgenes already in commercial use have been taken from viruses, bacteria, plants, and animals of various kinds.
The arrival of *Synthetic Biology* (see Section 6 of this Chapter) has created new potential opportunities to obtain useful genetic material for GE of organisms. It is now feasible to synthesize tailored versions of any gene, gene cluster or promoter.

1.3 Vector construction for gene transfer into higher organisms: general aspects

The introduction of foreign DNA into bacterial or yeast cells is called *transformation*. In animal cells the term *transfection* is used for the same process, in order to avoid confusion. The reason for this is that transformation refers to phenotypic changes taking place when cells are underway from being normal to becoming malignant cancer cells. For plant cells, both designations may be used. In the GE context, transformation and transfection relates to the same phenomena: inherited changes that are due to the introduction of foreign, exogenous DNA.

The process of expression vector construction is based on the same methods and tools as cloning vector construction. In principle, eukaryotic expression vectors do not differ from their prokaryotic counterparts. A basic eukaryotic expression vector must contain:

i) A eukaryotic promoter that secures the transcription of the transgene;
ii) A multiclonic site (MCS), i.e. a DNA sequence composed of recognition motifs for a number of restriction endonucleases;
iii) Eukaryotic transcriptional and translational stop signals;
iv) A DNA sequence that enables polyadenylation of the mRNA;

v) A selectable eukaryotic marker gene. The target gene can undergo a series of additions (e.g. insertion of specific promoter-intron combinations), deletions (of unwanted introns or codons), or other modifications (DNA sequence changes for preferential codon usage) to optimize for expression in the desired host.

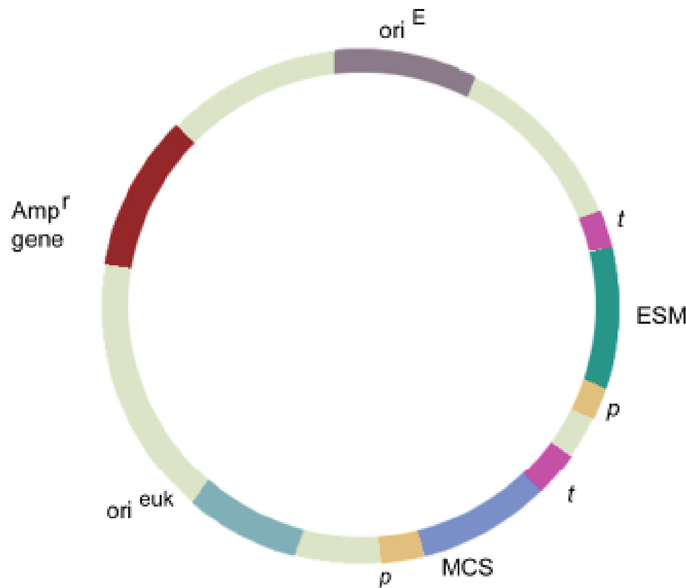

Figure 4.3. Outline of an eukaryotic expression vector. The major features are: promoter (p); the multiple cloning site (MCS) for a transgene, polyadenylation and termination signals (t); eukaryotic selectable marker gene (ESM); eukaryotic origin of replication (ori^{euk}); E. coli origin of replication (ori^{E}); an E. coli selectable marker gene (Amp^{r}).

Most eukaryotic vectors are shuttle vectors with two origin of replications and two selectable marker genes. One set functions in *E. coli*, the other in the chosen eukaryotic host cell. An outline of a generic eukaryotic expression vector is given in Fig. 4.3. It is important to emphasize that in real life there is no such thing as 'a generic expression vector'. All the elements inserted into a eukaryotic expression vector are carefully selected to be optimally functional in the target species and cell type of choice.

2. Insertion of genes into plants

Crop plants

There are three major reasons for developing transgenic plants. First, the addition of a gene(s) may improve the agricultural, horticultural, or ornamental value of a crop plant. Second, transgenic plants can act as living bioreactors for the inexpensive production of economically important proteins or metabolites. Third, plant genetic transformation (*transgenesis*) provides a powerful means for studying the action of genes during development and other biological processes.

Some of the traits that can be introduced into plants by a single gene construct or, possibly, a small cluster of gene constructs include: insecticidal activity, protection against viral infection, resistance to herbicides, protection against pathogenic fungi and bacteria, delay of senescence, tolerance of environmental stresses, altered flower pigmentation, improved nutritional quality of seed proteins, increased post-harvest shelf life, as well as self-incompatibility and male sterility and seed sterility. In addition, transgenic plants can be made to produce a variety of useful compounds, including therapeutic agents, polymers, and diagnostic tools such as antibody fragments. Alternatively, they can be engineered to synthesize viral antigenic determinants and, after ingestion, can be used as edible vaccines.

To date, over 140 different plant species have been genetically transformed, including many crop and forest species. Plant GE may have a big impact on plant breeding programmes because it promises to significantly reduce the 10 to 15 years that it takes to develop a new variety using traditional plant breeding techniques. Genetically modified (GM) plants are now prevalent in parts of the world and appear in processed food products worldwide.

Forest trees
The demand for wood is expected to grow by 20% in the next decade, while the world's forest cover declines at an annual rate of 9.4 million hectares, an area comparable to that of Portugal.[1] Breeding of trees is a slow process, partly due to their long generation time. Hence, it is conceivable that the utilization of transgenic trees or marker-assisted breeding may alleviate the gloomy prospects of the present.

Genomic sequencing projects and genome mappings have opened the road to transgenesis for several tree species, such as birch, pine, eucalyptus, spruce, oak, and acacia. The genus *Populus* (poplars) has been adopted as the model of choice due to advantages such as fast growth, amenability to tissue culture and genetic transformation, and a small genome (approximately 500 Mega base pairs).[2] Only China has reported the commercial release of transgenic poplar. In 2004 approximately 1.4 million insect-resistant trees were planted on 300–500 hectares. Insect resistance was achieved by transgenesis of *cry* genes from *Bacillus thuringiensis* (FAO, 2004). In the scientific community, GE of forest trees is considered an important avenue to domestication and increased yields. One of the arguments is that GE circumvents the long generation times that are typical for most forest trees. Most efforts so far have been devoted to improve lignin extraction during pulping. However, there have also been published promising results related to pathogen and pest resistance, bioremediation, acceleration and prevention of flowering, and herbicide resistance (for a review, see Boerjan, 2005). Most transgenic forest treelines obtained so far are derived from transformation of somatic embryonal tissues (somatic embryos) via co-cultivation with *Agrobacterium tumefaciens* (see below).

Fruit trees
Regeneration and transformation systems using mature plant material of woody fruit species have been achieved as a necessary requirement for trangenesis of cultivars. Once a useful transformant is isolated, unlimited production of the desired transgenic line can be achieved by *vegetative propagation*, the normal method of multiplying fruit trees. The only transgenic fruit tree being commercially produced at present is papaya (*Carica papaya*) resistant to PRSV (Papaya ringspot virus). In this case transformation was achieved by micoprojectile bombardment. More

[1](http://www.fao.org/forestry/site/28679/en/, accessed June 11, 2007).
[2]In addition to their value for wood products, members of the genus Populus provide a range of ecological services, including carbon sequestration, bioremediation, nutrient cycling, biofiltration, and providing diverse habitats, and this is the case for many other forest trees as well.

commonly, however, DNA has been transferred to fruit trees by disarmed and transgenic *Agrobacterium* strains. Recent overviews of transgenes and intended traits in fruit trees are now available (e.g. Petri & Burgos, 2005).

Vector considerations
There are a number of DNA delivery systems and expression vectors that work with a range of plant cells. Furthermore, most plant cells are *totipotent* – meaning that an entire plant can be regenerated from a single plant cell – so fertile plants that carry an introduced gene(s) in all cells (i.e. transgenic plants) can often be produced from genetically engineered tissue cultures. If the developed transgenic plant flowers and produces viable seed, the desired trait is passed on to successive generations.

Transformation with the Ti plasmid of Agrobacterium tumefaciens
The soil bacterium *A. tumefaciens* is a phytopathogen that, as a normal part of its natural life cycle, genetically transforms plant cells. This genetic transformation leads to the formation of crown gall tumours, which interfere with the normal growth of an infected plant. This agronomically important disease affects only *dicotyledonous plants (dicots)*, including grapes, stone-fruit trees (e.g. peaches), and roses.

Crown gall formation is the consequence of the transfer, integration and expression of genes of a specific segment of bacterial plasmid DNA – called the T-DNA (transferred DNA) – into the plant cell genome. The T-DNA is actually part of the 'tumour-inducing' (Ti) plasmid that is carried by most strains of *A. tumefaciens*. Depending on the bacterial strain that is host to the Ti plasmid, the length of the T-DNA region can vary from approximately 12 to 24 kilobase pairs (kbp).

Ti Plasmid-Derived Vector Systems
The simplest way to exploit the ability of the Ti plasmid to genetically transform plants is to insert the desired recombinant DNA sequence into the T-DNA region and then use the Ti plasmid and *A. tumefaciens* to deliver and insert this gene(s) into the genome of a susceptible plant cell. Although the Ti plasmids are effective as natural gene transfer vectors, they have several serious limitations as routine cloning vectors.

First, it is not possible to regenerate transformed cells into mature crop plants without prior removal of some genes contained in the Ti plasmid.
Second, naturally-occurring Ti plasmids are large (approximately 200–800 kb). For recombinant DNA experiments, however, a much smaller version is preferred, so large segments of DNA that are not essential for the cloning vector purposes are removed.
Third, because the Ti plasmid does not replicate in the bacterium *Escherichia coli*, the convenience of perpetuating and manipulating Ti plasmids carrying inserted DNA sequences in this laboratory bacterium does not exist. Therefore, in Ti plasmid-based vectors, an origin of replication that can be used in *E. coli* is added.
To overcome these constraints, recombinant DNA technology has been used to create a number of Ti plasmid-based vectors. These vectors are similarly organized and contain the following components:

(i) A selectable marker gene, such as the antibiotic resistance gene neomycin phosphotransferase (*nptII*), that confers kanamycin resistance to transformed plant cells. Because *nptII*, as well as many of the other marker genes used in plant transformation, is prokaryotic in origin, it is necessary to put it under the control of plant (eukaryotic) transcriptional regulation signals, including both a promoter and a

termination/polyadenylation sequence, to ensure that it is efficiently expressed in transformed plant cells.

(ii) An origin of DNA replication that allows the plasmid to replicate in *E. coli*. In some vectors, an origin of replication that functions in *A. tumefaciens* has also been added.

(iii) The right border sequence of the T-DNA region. This region is absolutely required for T-DNA integration into plant cell DNA, although most cloning vectors include both a right and a left border sequence.

(iv) A polylinker (MCS, multiple cloning site) to facilitate insertion of the cloned gene into the region between T-DNA border sequences.

Based on these alterations, a number of different Ti-plasmid based constructs have been used to bring recombinant genes into plant cell cultures from which mature plants may be regenerated. Two examples of such constructs are given in Fig. 4.4. Further developments include Ti-plasmid constructs designed to give recombinant gene expression in mitochondria or chloroplasts (see Section 6 of this chapter). The Ti-plasmids are propagated in *E. coli* before being transferred to *A. tumefaciens* for transformation of plant cell cultures.

Figure 4.4. A cloning vector derived from a Ti plasmid. This binary vector has origins of DNA replication (ori) for both E. coli and A. tumefaciens. A bacterial selectable marker gene can be used in both hosts. Both the transgene and the plant selectable marker gene are inserted between the T-DNA left and right borders.

Gene delivery methods

Although *A. tumefaciens*-mediated gene transfer systems are effective in several species, *monocotyledonous plants (monocots)*, including the world's major cereal crops (rice, wheat and maize), are not readily transformed by *A. tumefaciens*. However, by refining and carefully controlling conditions, protocols have been devised for the transformation of maize (corn) and rice by *A. tumefaciens* carrying Ti plasmid vectors. Many of the early plant transformation experiments were conducted with limited-host-range strains of *Agrobacterium*. However, more recently, wide-host-range strains that infect most plants have been tested and found to be effective, so many of the plant species that previously appeared to be refractory to transformation

by *A. tumefaciens* can now be readily transformed. Thus, when setting out to transform a new plant species, it is necessary to determine which *Agrobacterium* strain and Ti plasmid are best suited to that particular plant.

When the difficulties in transforming some plant species first became apparent, a number of procedures that could act as alternatives to transformation by *A. tumefaciens* were developed. Several of these methods require the removal of the plant cell wall to form *protoplasts*. Plant protoplasts can be maintained in culture as independently growing cells, or, with a specific culture medium, new cell walls can be formed and whole plants can be regenerated. In addition, transformation methods have been developed that introduce cloned genes into a small number of cells of a plant tissue from which whole plants can be formed, thereby bypassing the need for regeneration from a protoplast. At present, most researchers favour the use of either Ti plasmid-based vectors or microprojectile bombardment to deliver DNA into plant cells.

Microprojectile bombardment (also called *biolistics*), is the most important alternative to Ti plasmid DNA delivery systems for plants. Gold or tungsten spherical particles (approximately 0.4 to 1.2 micrometers (μm) in diameter or about the size of some bacterial cells) are coated with DNA that has been precipitated (with $CaC1_2$, spermidine, or polyethylene glycol). The coated particles are then accelerated to high speed (300 to 600 metres/second) with a special apparatus called a *particle gun (or 'gene gun')*. The original version of the gene gun used a small amount of gunpowder to provide the propelling force. The device that is currently used employs high-pressure helium as the source of particle propulsion (Fig. 4.5). The projectiles can penetrate plant cell walls and membranes; however, the particle density used does not significantly damage the cells. The extent of particle penetration into the target plant cells may be controlled by varying the intensity of the explosive burst; altering the distance that the particles must travel before reaching the target cells, or using different-sized particles.

Once inside a cell, the DNA is detached from the particles and, in some cells, integrates into the plant DNA. Microprojectile bombardment can be used to introduce foreign DNA into plant cell suspensions, callus cultures, meristematic tissues, immature embryos, protocorms, coleoptiles, and pollen in a wide range of different plants, including monocots and conifers, plants that are less susceptible to *Agrobacterium*-mediated DNA transfer. Furthermore, this method has also been used to deliver genes into chloroplasts and mitochondria, thereby opening up the possibility of introducing exogenous (foreign) genes into these organelles.

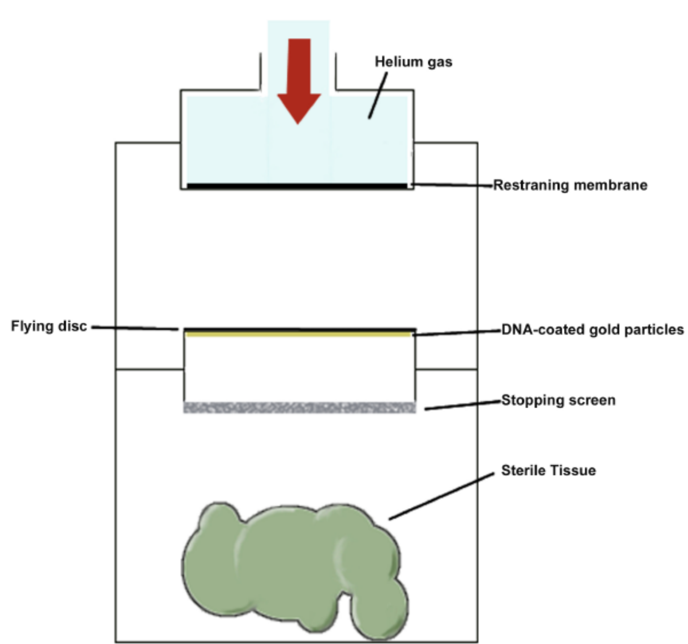

Figure 4.5. Schematic representation of a 'gene gun' (microprojectile bombardment apparatus). The plastic restraining membrane bursts when the helium pressure reaches a certain point. The released gas then accelerates the flying disk containing the DNA-coated gold particles on its lower side. The gold particles pass the stopping screen, while the flying disc is held back, and penetrate the cells of the sterile tissue.

Typically, plasmid DNA dissolved in a buffer is precipitated onto the surface of the microprojectiles. Using this procedure, it is possible to increase the transformation frequency by increasing the amount of plasmid DNA; however, too much plasmid DNA can be inhibitory. It is estimated that there are approximately 10,000 transformed cells formed per bombardment. With this technique, cells that appear to be transformed, based on the expression of a marker gene, often only transiently express the introduced DNA. Unless the DNA becomes incorporated into the genome of the plant, the foreign DNA may be degraded eventually.

The configuration of the vector that is used for biolistic delivery of foreign genes to plants influences both the integration and expression of those genes. For example, transformation is more efficient when linear rather than circular DNA is used. Moreover, large plasmids (>10 kb), in contrast to small ones, may become fragmented during microprojectile bombardment and therefore produce lower levels of foreign gene expression. However, large segments of DNA may be introduced into plants using *yeast artificial chromosomes (YACs)*. The YACs were engineered to contain plant selectable markers as well as yeast selectable markers. In a number of experiments the presence of distantly situated plant selectable marker genes in transformed plant cells indicates that the entire YAC, along with the entire inserted foreign DNA, was probably transferred. DNA hybridization experiments revealed that YACs up to 150 kbp in total size have a good chance of being stably integrated into the plant cell.

Use of reporter genes in transformed plant cells

It is essential to be able to detect the recombinant DNA that has been integrated into the plant genome so that those cells that have been transformed and are expressing the vector gene cassette can be identified. Furthermore, in studies of plant transcriptional regulatory signals and the functioning of these signals in specific plant tissues (such as leaves, roots and flowers), it is often important to be able to quantify the level of expression of a gene with a readily identified product.

Quantification and other applications require the use of *reporter genes* that either permit transformed cells to be selected or encode an activity that can be assayed. To these ends, a number of different genes have been tested as reporters for transformation, including genes that can be used as dominant selectable markers and genes whose proteins produce a detectable response to a specific assay.

Many of these reporter genes are from bacteria and have been equipped with plant-specific regulatory sequences for expression in plant cells. Dominant marker selection provides a direct means of obtaining only transformed cells in culture. For example, in the presence of the antibiotic kanamycin, only plant cells with a selectable marker gene *nptII* gene can grow.
The inclusion of marker genes encoding antibiotic resistance in transgenic plants has raised concerns. The antibiotic resistance genes that are used as selectable markers might be transferred to pathogenic soil or gut microorganisms. Moreover, it is possible that the products of some marker genes might be either toxic or allergenic. The presence of some reporter genes and their products may limit the market potential of the commercial product. To allay these concerns, strategies for the production of transgenic plants without any marker genes have been developed (Darbani et al., 2007).

Gene expression considerations
When genetic transformation of crop plants became routine, research efforts were directed toward introducing a wide range of recombinant plant and bacterial genes into plants. The transformed plants were assayed for the production of the foreign protein and studied physiologically to assess how the presence of an additional, novel protein affected the whole plant. Many of these early experiments utilized promoters that were expressed constitutively (i.e. they were always 'on', and not regulated) in a range of plant cells. More recently, many additional plant promoters have been isolated and characterized, and used to express foreign proteins in specific cells at certain times during the growth and development of the plant. For example, instead of the strong constitutive 35S promoter from cauliflower mosaic virus (*35S CaMV promoter*), which is expressed in all plant tissues and throughout the life of the plant, researchers have used the promoter for the small subunit of the photosynthetic enzyme ribulosebisphosphate carboxylase, which is active only in photosynthetic tissues such as leaves. Similarly, plant promoters active only in specific tissues, such as roots or flowers, or only during periods of environmental stress (e.g. the pathogenesis-related promoters), or in the presence of chemical inducers, have been used to control the expression of some foreign genes.

The level of expression of a foreign protein under the control of the 35S CaMV promoter is often lower than desired. To address this problem, it is necessary to test different promoter/gene constructs in plants to see if more effective promoters can be found.[3] In addition to the promoter, several other elements may enhance foreign gene expression. These include enhancer sequences (found from one to several hundred nucleotides upstream of the promoter sequence), introns (that may stabilize messenger RNA), and transcription terminator sequences (see Chapter 3 for further details).

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[3]In one series of experiments, recombinant DNA constructs that contained all or some of the following elements were tested: the 35S promoter, the nopaline synthase gene transcription terminator, from one to seven tandemly repeated enhancer elements, and a DNA sequence from tobacco mosaic virus called (omega) that increases gene expression at the translational level. The most active construct contained seven enhancer elements and directed a much higher level of foreign gene expression in both transgenic tobacco and rice plants than when the 35S promoter alone was used. These promoter constructs directed a wide range of foreign gene expression in different transgenic plant lines. This variation is probably due to the site within the plant genome where the T-DNA is inserted. Nevertheless, this work shows that it is possible to engineer promoters that are much stronger than the naturally occurring 35S promoter. With this approach, it should be possible to engineer promoters that are tissue specific, developmentally regulated, and robust.

3. Insertion of genes into animals

Applications for transgenic animals

As livestock animals and their products constitute a major factor in human nutrition, the purposeful genetic modification of livestock animals has special implications. Any additional safety risks introduced by the genome modification, whether real or only perceived, are highly unlikely to be accepted by either the regulatory authorities or consumers. Traditional GE approaches involve new recombination events between unrelated DNA sequences, something which has been considered as a potential risk and is currently limiting the acceptability of the technology.

The production of transgenic animals has focused mainly on producing models, for instance in the mouse, for basic and medical research. In terms of commercially important livestock species, work has revolved around specialized non-agricultural purposes such as pharmaceutical production and xenotransplantation. To a lesser extent, agricultural applications to improve animal production traits and food quality have also been pursued. The first reports of transgenic livestock came in the 1980s, and focused on introducing growth-promoting genes into pigs and sheep. The present century, however, has already seen transgenic swine (EnviroPigs) carrying a bacterial phytase gene driven by a salivary gland-specific promoter. Phytase breaks down phosphates in the pigs' feed, reducing phosphorus excretion in the manure by up to 75%, and thus reduces environmental pollution (Golvan et al., 2001).

In spite of the low efficiency of the microinjection methods, a number of transgenic livestock have already been established, e.g. pigs with growth hormone transgenes and sheep with keratin-IF-I transgenes for improved wool quality. 'Transgenic animal bioreactors' are based on the fact that animal cells are required to synthesize proteins with the *appropriate post-translational modifications* (see Chapter 3). Transgenic animals are being used for this purpose. Milk, egg white, blood, urine, seminal plasma, and silk worm cocoons are candidates for the sources of recombinant proteins at an industrial scale.

Transgenesis to engineer disease-resistant livestock is another goal pursued. Mastitis (mammary gland infections) costs the US dairy industry approximately USD 2 million annually, and has a similar impact in Europe. *Staphylococcus aureus* is a major mastitis pathogen, and it is highly sensitive to lysostaphin. Lysostaphin-transgenic cattle, expressing the antimicrobial peptide in their mammary epithelium, excrete the product in their milk. Transgenic cows resist *S. aureus* mammary gland infections, and their milk kills the bacteria in a dose dependent manner (Wall et al., 2005).

The process of evaluating transgenic pigs as potential donors for *xenotransplantation* to humans involves a number of complex steps. It is one of the most widely discussed applications of transgenesis and cloning, although it does not seem to be a viable choice in the near future (Vajta & Gjerris, 2006).

Transgenic chickens could be used to improve the genetic make-up of existing strains with respect to built-in (in vivo) resistance to viral, bacterial and coccidial diseases, better feed efficiency, lower fat and cholesterol levels in eggs, and better meat quality. Avian researchers have also suggested that the egg, with its high protein content, could be used as a source of pharmaceutical proteins. By analogy to the mammary glands of livestock, the expression of a transgene in the cells of the reproductive tract of a hen that normally secretes large amounts of ovalbumin could lead to the accumulation of a transgene-derived protein that becomes encased in the eggshell. The recombinant protein could either be fractionated from these sterile packages or

consumed as a nutraceutical with breakfast. The expected annual yield of recombinant protein from one hen is 0.25 kg. Currently, as 'proof-of-principle' experiments, transgenic chickens that synthesize monoclonal antibodies, growth hormones, insulin, human serum albumin, and alpha-interferon have been created. Production of germ line transgenic chickens has also been achieved by using a retrovirus-based vector (Koo et al., 2006).

Vector and gene delivery considerations
Microinjection of foreign DNA (*expression plasmids*, see Section 1.3) into pronuclei of zygotes has been the method of choice for the production of transgenic domestic animals. This method is simple, but very inefficient because
(i) a large number of embryos are lost and
(ii) gene transfer rates are very low.

The low transgenesis rates result in enormous production costs. A transgenic cow would come with a price tag of at least USD 300,000 (Wells et al., 1999).

Following microinjection *the transgene is randomly integrated into the host genome*, which can be associated with insertional mutagenesis, unpredictable expression levels of the transgene and unwanted pathological side effects. However, a systematic analysis of potential pathological side effects putatively associated with the random integration and expression of a specific transgene in transgenic domestic animals has not yet been reported (Deppenmeier et al., 2006).

The need for a better method of livestock transgenesis was a major driving force behind the development of *nuclear transfer technology* that led to the generation of Dolly the sheep. In the following years, methods to introduce transgenes into the germ line of various animal species were presented, but they were often too inefficient and costly for practical applications. *Nuclear transfer (cloning)* is a possible way to generate transgenic animals in different species. However, this approach is both difficult and burdened by extremely high failure rates. The vast majority of clones die at various stages of development or shortly after birth. This phenomenon has been termed 'cloned offspring syndrome', and seems to be due to faulty e*pigenetic programming* of the donor genome (Vajta & Gjerris, 2006).

Hence, the use of *lentivirus vectors* for introduction of transgenes into the germ line (see the following) was a major breakthrough, which now seems to make production of transgenic livestock for agricultural and medical purposes feasible (Maga, 2005).

Viral vectors and delivery vehicles
Viral vectors can be divided into two groups according to the basic life cycles of their parental viruses. They are either *non-integrating* or *integrating*. Only the latter can be used for transgenesis, because the genomes of the former would be lost during the cell divisions of early embryonic development.

The majority of available integrating viral vectors are based on representatives of the large family *Retroviridae*. *Lentiviruses*[4] belong to this family. Recently, a lot of attention and effort has been focused on the construction of non-integrating expression vectors, but the most promising vector systems at present seem to be based on various lentivirus-based constructs (Jackson et al., 2006;

[4]Lentiviruses have been isolated from sheep (visna/maedi virus), goats (caprine arthritis encephalitis virus), cattle (bovine immunodeficiency virus), horses (equine infectious anemia virus, EIAV), cats (feline immunodeficiency virus), monkeys (simian immunodeficiency virus), and humans (human immunodeficiency virus, HIV). The best studied example of a lentivirus is HIV.

Vajta & Gjerris, 2006.) All retroviruses contain single-stranded RNA genomes. They carry with them a viral enzyme, reverse transcriptase, which transcribes the RNA genome into a DNA copy that is made double stranded (ds) by a DNA polymerase. During infection of host cells, the dsDNA is integrated into the host genome as a provirus, and serves as a template for the production of progeny virus particles.

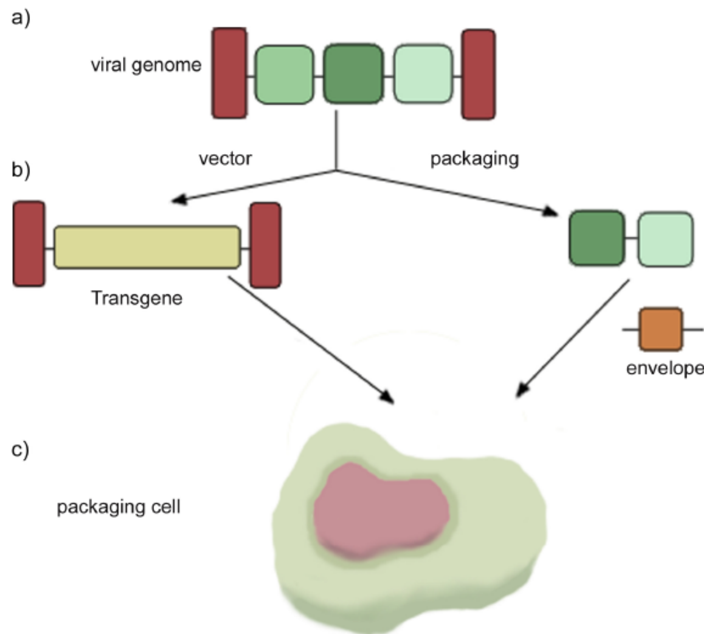

Figure 4.6. The basic principle of lentivirus vector construction. (a) Lentivirus genome depicting viral genes surrounded by LTRs (long terminal repeats) with their promoter and other cis-acting elements. (b) Division of the viral genome into a vector, containing the transgene, and a packaging unit. A gene expressing a suitable envelope (env) protein is supplied to broaden the spectrum of cells that accept vector infection. The packaging cells (c) express the viral proteins necessary for production of infectious virus particles in trans.

The basic concept of viral vector development is simple (Fig. 4.6) (for a review see Pfeifer, 2004): (i) identify viral genes relevant for pathogenesis, replication and production of infectious virus particles; (ii) delete all viral protein coding sequences; (iii) incorporate the transgene in the viral vector; and (iv) produce virus particles that carry the vector genome in packaging helper cells that provide essential viral proteins in *trans*. The vector virus particles are replication defective. During their use in a transgenic process the vector genome can only carry out a single round of infection. Hence, the integrated proviruses cannot produce progeny virus, but its genes can be efficiently transcribed by the host cell transcriptional system.

An important safety concern with lentivirus vectors is the possibility of insertional activation of cellular *oncogenes* by random integration of the vector into the host genome (see Chapter 8). The newest generation of such vectors are therefore self-inactivating (SIN). This is achieved by deletion of the lentivirus enhancer and promoter sequences, leaving the transgene promoter as the only transcriptionally functional element (Pfeifer, 2004).

Lentivirus transgenesis has been achieved for mice, rats, pigs, cattle, and chickens. In swine, infection of early zygotes with lentivirus-vectored transgenes has given high frequency of stably transgenic piglets. Zygote infection has not worked out well for cattle, while infection of bovine

oocytes before *in vitro* fertilization has been successfully carried out. Lentivirus vectors can also be used to *transfer transgenes into cells before their nuclei are transferred into enucleated bovine oocytes*. Bovine foetal fibroblasts have been used as nucleus donors (Hofmann et al. 2003; 2004). *RNA interference (RNAi)* has recently emerged as a novel method to knock down gene expression in mammalian cells. Lentiviral vectors carrying promoter-driven expression of short inhibitory (si) RNAs have recently been shown to induce efficient gene silencing in mice (Rubinson et al., 2003). Lentiviral RNAi vectors may prove valuable for gene expression knock down in farm animals as well. Such vectors might be used therapeutically to inhibit expression of disease-promoting genes (Pfeifer, 2004).

Cloning livestock by nuclear transfer
In a highly publicized case, a sheep named Dolly was cloned by transfer of a nucleus from a mammary (udder) cell of an adult organism. This was the first demonstration of pluripotency (totipotency) of a nucleus of a differentiated adult cell. Since the cloning of Dolly, somatic cell nuclei have been used to clone cattle, goats and pigs. In these cases, the nuclear transfer procedures are similar (Fig. 4.7). Briefly, embryonic, foetal or adult donor cells are isolated, cultured and genetically modified. Although not always feasible with adult cells, prolonged culture is preferred because experimenters have additional time to carry out successive genetic alterations, such as inactivating both alleles of a locus or creating multiple gene changes. After establishing a cell line with a specific genetic modification(s), individual donor cells are fused to an enucleated oocyte with short-duration electric pulses. For example, two 2.5 kilovolts per centimetre (kV/cm) pulses for 10 microseconds each are used to fuse adult cattle fibroblast cells with enucleated oocytes. The pulses simultaneously induce cell fusion and oocyte activation. Each fused cell is cultured to the blastocyst stage before being transferred into the uterus of a pseudopregnant female. At birth, genotype analysis is used to confirm the presence of the transgene.

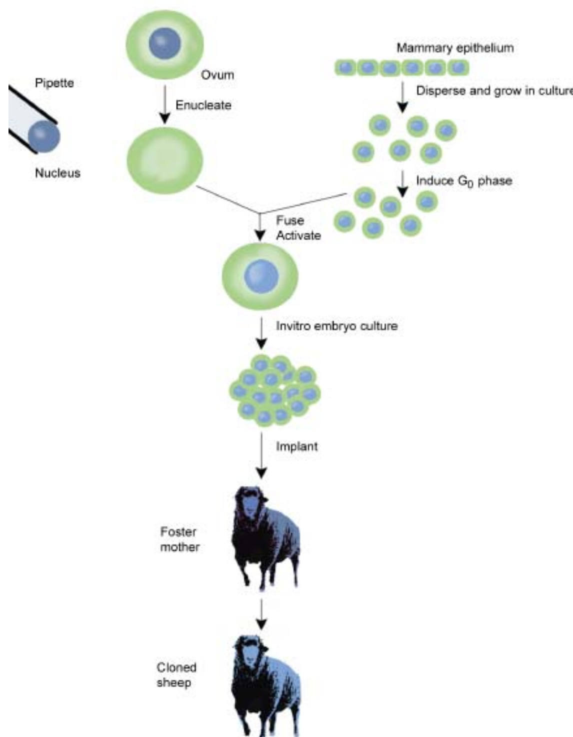

Figure 4.7. Cloning of sheep by nuclear transfer.

Transgenic fish

As natural fisheries become exhausted, production of this worldwide food resource will depend more heavily on aquaculture. In this context, enhanced growth rates, tolerance to environmental stress and resistance to diseases are some of the features that may be created by transgenesis. To date, transgenes have been introduced by microinjection or electroporation of DNA into the fertilized eggs of a number of fish species, including carp, catfish, trout, salmon, Arctic char, and tilapia. The pronuclei of fish are not readily seen under a microscope after fertilization; therefore, linearized transgene DNA is microinjected into the cytoplasm of either fertilized eggs or embryos that have reached the four-cell stage of development. Unlike mammalian embryogenesis, fish egg development is external; hence, there is no need for an implantation procedure. Development of transgenic fish occurs in temperature-regulated holding tanks. The survival of fish embryos after DNA microinjection is high (35% to 80%), and the production of transgenic fish ranges from 10% to 70%. The presence of a transgene is scored by PCR analysis of either nucleated erythrocytes or scale DNA. Founder fish are mated to establish true-breeding transgenic lines. Many of the initial studies with transgenic fish have focused on examining the effect of a growth hormone transgene on growth rate. In one study, a transgene consisting of the promoter region from the antifreeze protein gene of the ocean pout (*Zoarces americanus*), the growth hormone cDNA from salmon, and the termination-polyadenylation signals from the 3' end of the antifreeze protein gene from the ocean pout were injected into eggs of Atlantic salmon. In general, the transgenic salmon were larger and grew faster than the non-transgenic controls. This expression system was chosen to enhance the transcription of the growth hormone in cold waters. An 'all-fish' construct was assembled to avoid possible biological incompatibilities that might arise from using a growth hormone gene from non-fish sources. For even greater specificity an 'all-salmon' growth hormone construct was formulated and microinjected into sockeye salmon eggs. After approximately one year, the transgenic salmon were approximately eleven times heavier than the non-transgenic salmon. However, there was no difference in size between adults. Theoretically, the faster growth of young salmon would lower the cost of the feed and lessen the pollution of coastal waters in the vicinity of the holding pens. There is the further possibility that aquaculture with transgenic fish can be carried out within contained facilities. Regardless, the full impact of the accidental release of transgenic fish on natural populations must be considered if they are raised in ocean pens. Genetically engineered fish with enhanced phenotypic traits have yet to be implemented into commercial applications. This is partly because of the difficulties in reliably predicting the ecological risk of transgenic fish should they escape into the wild (Devlin et al., 2006).

4. Insertion of genes into microorganisms

Applications for transgenic microorganisms

A high number of bacteria and yeasts have been genetically engineered for production of industrially, nutritionally and medically important eukaryotic gene products under *contained* conditions. Yeasts, e.g. *Saccharomyces cerevisiae* and *Pichia pastoris*, are often the organisms of choice for such purposes. The reasons for this are mainly that bacteria do not carry out the post-translational modifications of transgenic proteins that are necessary for their authentic structure and proper functioning. Consequently, yeasts have been used to produce recombinant proteins from eukaryotic genes. A number of bacteria have, however, been made transgenic for the purposes of *environmental bioremediation* and as *probiotics*. The use of such genetically engineered bacteria implies direct or indirect release to the environment.

Sites contaminated by metals (e.g. Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Cr^{2+}) and *xenobiotics* (e.g. trichloroethylene, PCBs (polychlorinated biphenyls), dioxins, trinitrotoluene, PAHs (polycyclic

aromatic hydrocarbons), nitroglycerine) pose enormous health and environmental problems. At present, contaminated sites are treated by physical, chemical and thermal processes following excavation and transportation. The cost of removal of one m^3 of contaminant from a one acre contaminated site is estimated at USD 0.6–2.5 million. In contrast, the cost of bioremediation by transgenic plants or microorganisms is estimated at USD 2–5000. In addition, bioremediation causes minimum site disruption, stabilizes the soil against erosion, and concentrates heavy metals (Davison, 2005).

Naturally occurring microorganisms capable of degrading a variety of toxic compounds under laboratory conditions have been isolated. However, as many of the xenobiotic pollutions are novel to the ecosystems, microorganisms have not evolved appropriate metabolic pathways to degrade them. This is where transgenic microorganisms may fill a void in bioremediation strategies (Pieper & Reineke, 2000).

Probiotics (the name is derived from the Greek 'for life') have been used for *c.*100 years to treat a variety of mucosal surface infections, such as those of the gut and vagina, but the use of these traditional treatments diminished after the advent of antibiotics. However, these agents are now being reconsidered as alternatives to antibiotics because of the rise in antibiotic-resistant strains of bacteria. Probiotics have many potential therapeutic uses, but have not been universally accepted because of a lack of understanding of their action. Lactic acid bacteria (LAB) have been modified by traditional and GE methods to produce new varieties. Modern techniques of molecular biology have facilitated the identification of probiotic LAB strains, but only a few LAB have been modified by recombinant-DNA technology because of consumer resistance to their introduction to markets, especially in Europe (Ahmed, 2003; Celec et al., 2005).

Vector considerations
A eukaryotic gene will not function in a prokaryotic organism because there is no mechanism for removing introns from transcribed RNA. Furthermore, a eukaryotic gene needs prokaryotic transcriptional and translational control sequences to be properly expressed. Special strategies are therefore required for cloning and expressing eukaryotic coding regions in prokaryotic cells. The intron problem is overcome by the synthesis of cDNA copies of functioning mRNAs, and the necessary control sequences are added by the 'cut-and-paste' techniques described in Section 1.3. In addition to expression plasmids, other vectors that allow for cloning and expression of larger DNA fragments than plasmids can cope with are available for bacterial systems. *Bacteriophage λ vectors* for use with *E. coli* can accept inserts in the range of 15–20 kbp, while *cosmids* can cope with up to 45 kbp. Cosmids combine the properties of plasmids and λ vectors.

Gene delivery methods
Transformation is the process of introducing expression vectors into bacterial cells. Uptake of DNA is usually achieved either by *$CaCl_2$ precipitation* or by *electroporation.* The former method implies treatment of the cells with ice-cold $CaCl_2$ followed by exposure to high temperature (42°C).

Electroporation is based on the fact that uptake of free DNA can be induced by exposing bacteria to a high-voltage electric field. The expression is a contraction of the descriptive phrase 'electric field-mediated membrane permeabilization'.

For some bacteria, *conjugation*, the natural system of transmitting plasmids from one bacterial strain to another, has been used to transport an expression plasmid from a donor cell to a recipient cell that is not easily transformed by the aforementioned techniques.

Gene expression considerations
There is no single strategy to obtain optimal expression of every cloned gene. The features that are being manipulated to modulate gene expression include the promoter and terminator sequences, the strength of the ribosome-binding sites, the number of transgene copies, and whether the transgene is to be plasmid-borne or integrated into the host cell genome. Furthermore, it is important to consider the final cellular location of the transgene product, the efficiency of translation and the intrinsic stability of the product in the host cell. Most cloned genes have distinctive properties that require considerable time and effort before the optimal expression level is found.

Insertion precision
For GE bacteria that are to be, or that may indirectly become, released into the environment it is essential, for efficiency as well as biosafety reasons, that the transgenic DNA be neither easily lost or transferred to other organisms, e.g. by HGT (horizontal gene transfer; see Chapter 13). The integration of the transgenic construct into the bacterial genome may prohibit both loss and unintended spread of DNA.

For integration of the transgenic construct DNA into a chromosomal site, the input DNA must share some sequence similarity with the chromosomal DNA, and there must be a physical exchange, homologous recombination (HR), between the two DNA molecules. It was initially thought that recombination required at least a sequence similarity of some 50 nucleotides for HR to occur. This has, however, been shown not to be mandatory, a fact that opens the way for the integration of additional construct copies, or part of copies, in untargeted locations in the recipient genome (Ikeda et al., 2004; see also Chapter 13 and references therein).

5. Location of the inserted genes

Random insertions
The transgene DNA may integrate into or adjacent to plant genes and perturb their expression by either decreasing or increasing their expression. The transgene could be expressed in an unanticipated manner through actions from promoters in adjacent plant genes or via interactions of plant gene open reading frames (ORFs) with promoter elements in the plant transgene. Transgene rearrangements during integration can create spurious open reading frames (ORFs) and spurious ORFs could allow the transgene to produce unintended gene products. Recombination due to repeated sequences in the transgene may result in intralocus instability or may lead to ectopic recombination. Furthermore, effects of gene silencing can interfere with the desired gene expression (Haslberger, 2006). These and other areas of scientific ignorance and knowledge gaps of importance to risk assessment and management are further discussed in Chapter 8.

Gene targeting
Many scientists now recognize the unavoidable and unpredictable consequences of the present methods for transgenesis, whether based on naturally occurring or synthesized DNA/RNA. Hence**,** strategies to perform gene targeting, i.e. to insert the gene construct into a predetermined location in the genome have been pursued. This has been achieved, at a very low efficiency, by *homologous recombination* (HR) strategies. The purpose is to perform precise, site-specific modifications of the genome to introduce, functionally delete or subtly alter target genes or their regulatory sequences. Homologous recombination is, however, an extremely rare event in mammalian cells. Furthermore, although transfected gene constructs may find their predetermined sites, other copies of the construct may integrate randomly into other locations of the genome.

Superior phenotypic characteristics in livestock have been linked to quantitative trait loci (QTL). Many QTL are associated with point mutations, single-nucleotide polymorphisms. Hence, for genetic improvement of livestock, *oligonucleotide-mediated gene modification (OGM)* may be a safer and more acceptable strategy than GE transgenesis or HR-based approaches (Laible et al., 2006).

The OGM techniques are based on single-stranded oligonucleotides (ssODNs). They contain mismatches with regard to the target gene in the recipient genome. Upon transfection into the animal cell, the mismatches are introduced into the genomic target sequence. This in turn will give a changed or 'improved' protein product from the targeted gene. Thus, this is an approach that avoids some of the potential biosafety concerns related to the insertional mutagenesis results that may arise from untargeted integration of transgenes. At present, this technology is far from efficient enough for livestock animal applications, but future development and refinement may change this situation.

6. Future prospects for gene transfer methodologies

Gene 'stacking'

Most organismal characteristics and traits are the result of the cooperation between a number of genes. Hence, in order to obtain useful changes, a cluster of transgenes has to be transferred to the recipient organism. Progress towards second and third generation genetically modified organisms (GMOs), with nutritional, environmental or other benefits that consumers may appreciate, has been slow, and will continue to be so until the bottleneck of having methods to manipulate multiple genes or traits has been removed. The theoretical potential for sophisticated metabolic engineering in plants is enormous, and could lead to the development of plants able to grow in inhospitable environments, and provide healthier foodstuffs and improved raw materials. Similar statements have been made for transgenic animals. However, most metabolic processes that are targets for manipulation depend on the interaction between numerous genes. Hence, effective metabolic engineering will only be achieved by controlling multiple genes in the same, or interconnected, biochemical pathways (Halpin, 2005). For instance, three carotenoid biosynthesis genes have been engineered into 'Golden rice' to make it produce provitamin A. Efficient provitamin A absorption may, however, require that the resorbable iron content is enhanced. This will necessitate the introduction of three additional transgenes.

Significant progress in multigene transgenesis has been made during the last few years. A variety of conventional and new techniques has been employed. Despite imperfections, plant biotechnologists consider that they provide a promising framework for future improvements. Two or more genes can be sequentially introduced into an organism by conventional iterative procedures. A plant containing one transgene can be crossed with individuals harbouring other transgenes, or it is re-transformed by new transgenes. For example, crossing plants expressing different Bt toxins (cry genes) can provide an efficient way to delay the emergence of Bt-resistant pests. Yet despite some success stories, the iterative strategies for obtaining multi-transgenic plants have several significant limitations. Principal among these is the fact that the trangenes will not be linked, and will be sited in different random loci in the recipient genome. Furthermore, the procedures will be very costly and slow. Finally, a high number of selectable marker genes will be necessary, and this will not be easily accepted by regulatory authorities and the public. Although several strategies have been developed to remove marker genes, these are not foolproof and this may hinder the acceptance of such multi-transgene organisms.

Alternative strategies for obtaining multi-transgenic plants are now being exploited. These include co-transformation with multiple independent transgenes and 'linked effect transgenes'. The latter refers to two or more 'effect genes', each with its own promoter and terminator, that are positioned contiguously on DNA that will transfer as a single entity into the recipient genome, e.g. on a single T-DNA for *Agrobacterium*-mediated transformation. All these procedures are, however, limited by the fact that it is not possible to ensure that the transgenes are expressed at similar levels, even when they are physically linked. Ways to overcome these difficulties are sought through constructing polycistronic transgenes, polyprotein expression systems and chimeric transgenes for multiple gene expression (Halpin et al., 2005).

The 'stacking' of transgenes in crops offers the potential to provide multi-toxin resistance to particular pests, nutritional value enhancement, resistance to biotic and abiotic stress, and bioremediation of xenobiotics. Plant raw materials, such as fibres, oils and starch, may be produced more cost-effectively and be environmentally benign for processing by industry. Entirely new industrial and therapeutic products may be produced in crops in a substantial manner. Edible plant vaccines may offer immunologically superior and cost-effective alternatives to traditional vaccines (Singh et al., 2006).

Chloroplast transgenesis

In nuclear transgenic plants, expression of multiple genes requires introduction of individual genes and time-consuming subsequent backcrosses to reconstitute multi-subunit proteins or pathways, a problem that is compounded by variable expression levels, as well as unpredictable insertion sites, expression levels and genome stability of the transgenic plants. In order to accomplish expression of multiple genes in a single transformation event, several genes can now be introduced into the chloroplast genome.

In plant and animal cells, the *monocistronic translation* of nuclear messenger RNAs (mRNAs) that contain only one translational unit poses problems in engineering multiple genes in plants. In contrast, most chloroplast genes of higher plants are co-transcribed. Multiple steps of chloroplast mRNA processing are involved in the formation of mature mRNAs. Expression of *polycistrons* via the chloroplast genome provides a unique opportunity to express entire pathways in a single transformation event. Additionally, chloroplast GE, according to its proponents, is an environmentally friendly approach resulting in containment of foreign genes and hyperexpression (Bogorad, 2000).

Chloroplast GE is rapidly becoming the transformation method of choice for the next wave of transgenic products in crop plants, particularly for plant-made pharmaceuticals (PMPs). Chloroplast GE has been designed in order to obtain high levels of gene expression needed for target protein production, which can be up to 45% of the total soluble proteins produced in the cell (De Cosa et al., 2001), while limiting the amount of vertical gene flow from the maternally inherited chloroplasts.

Artificial chromosomes: YACs, BACs and MACs

Artificial chromosomes are DNA molecules of predictable structure which are assembled in vitro from defined constituents that are similar to natural chromosomes. The first artificial chromosomes have been constructed in yeast (*Saccharomyces cerevisiae*). They include *centromeres*, *telomeres*, and *origins of replication* as essential components. These yeast artificial chromosomes (YACs) can be introduced into cell lines. They carry much larger amounts of DNA than usually can be employed in microinjection. Microinjection of a 450 kb genomic YAC harbouring the murine tyrosinase gene resulted in transgenic mice which showed position independent and copy number dependent expression of the transgene. Lactoglobulin and human

growth factor were expressed in the mammary gland of transgenic rats. Artificial chromosomes can also be constructed in bacteria (BACs), which can be genetically modified more easily. Transgenic mice were generated via pronuclear injection of BACs and germ line transmission and proper expression of the transgene was achieved. However, to date, transgenic livestock have not been reported upon transfer of a YAC construct. This may be attributed to the inherent problems of this technology, such as difficulties in isolating YAC DNA with sufficient purity and the inherent instability with a tendency for deleting regions from the insert.

Mammalian artificial chromosomes (MACs) have been engineered by employing endogenous chromosomal elements from YACs or extra chromosomal elements from viruses or BACs and P1 artificial chromosomes (PACs). MACs with a size of 1–5 Mb were formed by a *de novo* mechanism and segregated like normal chromosomes upon introduction into cell lines. A human artificial chromosome (HAC) containing the entire sequences of the human immunoglobulin heavy and light chain loci has been introduced into bovine fibroblasts, which were then used in nuclear transfer. Transchromosomal offspring were obtained that expressed human immunoglobulin in their blood.

Satellite-DNA based artificial chromosomes (SATAC) are neochromosomes that are formed by *de novo* amplification of pericentric heterochromatin yielding chromosomes from 10 to 360 megabases. These can serve as chromosomal vectors for exogenous DNA. Transgenic mice have been generated by microinjection of SATACs into pronuclei of zygotes. The additional chromosome showed germ line transmission over three generations. Microinjection of SATACs was also compatible with the development of bovine embryos. Transgenic embryos could be identified by staining for the presence of a reporter gene and FISH detection of the extra chromosome.

Synthetic biology (see the following) offers new opportunities to make useful forms of artificial chromosomes.

Nanobiotechnology (NBT)
The size domain of nanotechnology is a billionth of a metre. Nanobiotechnology is thus defined as the use of nanoscale or nanostructured objects in the size range of 1 nm (nanometer) to 100 nm. *Nanocarriers* are materials or devices of nanoscale made up of different biodegradable materials such as natural or synthetic polymers, lipids or phospholipids, and even organometallic compounds. They offer attractive solutions for DNA transformation of cells and organisms. There are, however, a number of unsolved health and environmental biosafety issues related to the use of nanocarriers as gene delivery vectors (Hoet et al., 2004).

Synthetic biology
Synthetic biology is interpreted as the engineering-driven building of increasingly complex biological entities for novel applications. Some scientists even predict that the first man-made cell, capable of replication and evolution, fed only by small molecule nutrients, is now possible within the next decade or so (Forster & Church, 2006). Two of the synthetic biology application areas most significant for engineering of transgenic organisms are represented by *artificial gene networks* and *de novo synthesis of large DNA sequences*. Genomic-scale DNA synthesis is already becoming increasingly possible today. Furthermore, DNA synthesizing of an entire intracellular pathway, composed of genes from various species, is becoming feasible. Such approaches will include optimal codon usage, adapted secondary mRNA structures, tailored regulatory elements (e.g. promoters, enhancers, introns), and MCS strategies that allow the modular replacement of specific genes by improved versions (Heinemann & Panke, 2006). At this point, it is important to emphasize the fundamental difference between engineering in biology and

in, for instance, chemistry or physics. Biological systems have the capacity to replicate and to evolve. This may interfere with the short- and long-term stability of engineered pathways, constructs and organisms, and will require constant monitoring of the integrity of the systems.

RNAi technology
In addition to the traditional strategies for vector construction and genetic modification strategies described, RNAi (interference) technology (see Chapter 3) is now becoming a new way to improve the contents and fight the diseases of crop plants (Sen & Blau, 2006). Furthermore, plant virus vectors for transfer and expression of transgenes in crop plants are coming into use (Chung et al., 2006).

Hybrid technologies
It seems quite safe to predict the future development of transgenic organisms based on fusions and hybrids between transgenesis, nanobiotechnology, RNAi technology, and synthetic biology. Such developments will include tailored single transgenes, multimodular DNAs or artificial chromosomes more efficiently delivered to cells and organisms by different types of nanocarriers. The nanocarriers may be loaded with protein ligands that target the DNA constructs to specific cell types and facilitate the transport from the cell surface to the nucleus, and stable integration into the recipient cell genome.

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