3

Techniques for plant transformation

INTRODUCTION

Plant transformation has become widely adopted as a method both to understand how plants work and to improve crop plant characteristics. Plant transformation depends on the stable introduction of transgene(s) into the genome of the plant. Various methods have been developed to achieve this and many plant species have been transformed successfully. Some transformation methods are based on utilizing *Agrobacterium*, a pathogen of dicotyledonous (broad-leafed) plants that transfers genes into the plant genome. Problems with using *Agrobacterium* to transform monocotyledonous plants (grasses) spurred on the development of other methods, the so-called direct gene-transfer methods. The major direct gene-transfer method, particle bombardment (or biolistics), is the method of choice in many laboratories for the transformation of monocotyledonous plants, despite *Agrobacterium*-based protocols having subsequently been developed for the transformation of monocotyledonous plants.

In this chapter, we will look at both the biology of *Agrobacterium tumefaciens* and how it is used in plant transformation studies. Direct gene-transfer methods for plant transformation, and in particular biolistics, will also be considered and examples of their use for the transformation of monocotyledonous and dicotyledonous crops will be given.

Agrobacterium-mediated gene transfer

The biology of *Agrobacterium*

Agrobacterium tumefaciens is a soil-borne, Gram-negative bacterium, much beloved of plant biologists and biotechnologists alike. The explosion of interest in *A. tumefaciens* has been driven by several observations that have both biotechnological and purely scientific ramifications. *A. tumefaciens* is the causative agent of crown-gall disease, an economically important disease of many plants (particularly grapes, walnuts, apples, and roses). The ability to cause crown galls (tumorous tissue growths) depends on the ability of *Agrobacterium* spp. to transfer bacterial genes into the plant genome. This startling feature is, to date, one of the very few examples of inter-kingdom gene

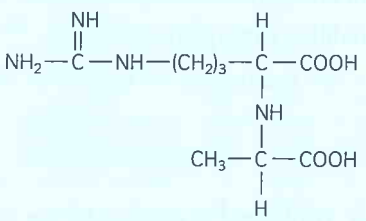
transfer, which has obvious scientific interest. More important to the biotechnologist is the development of plant transformation methods that make use of this unique capability.

Crown-gall disease

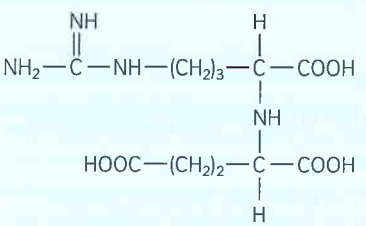
In order to fully appreciate and understand how *A. tumefaciens* became such a widely used tool in plant biotechnology it is worth looking at the biology of crown-gall disease. *A. tumefaciens*, the causative agent, is a Gram-negative, rod-shaped, motile bacterium found in the rhizosphere (the region around the roots of plants) where it normally survives on nutrients released from plant roots. However, if a plant is wounded or damaged, *A. tumefaciens* can infect the plant at the wound site and cause disease symptoms. *A. tumefaciens* is attracted to the wound site via chemotaxis, in response to chemicals (sugars and phenolic molecules) released from the damaged plant cells.

Plasmid DNA is transferred from *Agrobacterium* into the plant cell DNA, causing crown-gall disease.

Crown-gall formation depends on the presence of a plasmid in *A. tumefaciens* known as the Ti plasmid (Ti stands for tumour-inducing). Part of this plasmid (the transfer DNA, or T-DNA region) is actually transferred from the bacterium into the plant cell, where it becomes integrated into the genome of the host plant. The T-DNA carries genes that encode proteins involved in both hormone biosynthesis (of auxin and cytokinin) and the biosynthesis of novel plant metabolites called opines and agropines. The production of auxin and cytokinin causes the plant cells to proliferate and so form the gall. The proliferating cells also produce opines (which are amino acid derivatives) and agropines (sugar derivatives), which are used by *A. tumefaciens* as its sole carbon and energy source (Figure 3.1 shows the structure of the common opines, octopine and nopaline). Different strains of *A. tumefaciens* contain different Ti plasmids that code for the production of different opines. Opines and agropines are not normally part of plant metabolism and are very stable chemicals, which provide a carbon and energy source that only *A. tumefaciens* can use (genes on the Ti



Octopine



Nopaline

Figure 3.1 The structures of the opines octopine and nopaline are shown. Both octopine and nopaline are amino acid derivatives. Octopine is derived from the amino acid arginine and pyruvate. Nopaline is derived from arginine and α-ketoglutarate.

plasmid that are not transferred to the plant encode proteins involved in opine uptake and catabolism). *A. tumefaciens* has therefore developed the ability to genetically transform plant cells to usurp the plant's biosynthetic machinery and produce nutrients that only it can utilize.

The Ti plasmid

As the Ti plasmid has a central role in crown-gall formation, and it is a portion of the Ti plasmid (the T-DNA region) that is actually integrated into the genome of the host plant, we shall look at the Ti plasmid in more detail.

As we have seen, the ability of *A. tumefaciens* to cause crown-gall disease was found to depend on the presence in the bacterium of a large (≈200 kb) plasmid termed the Ti plasmid. Analysis of the nuclear DNA from plant tumours showed that a portion of the Ti plasmid was integrated into the genome of the host plant. This portion was termed transfer DNA (T-DNA) and was found to be responsible for the tumorous phenotype. Analysis showed that one or more copies of the T-DNA could be integrated into the genome, but, in general, the T-DNA insertions in the plant genome were bordered by small (24 bp), nearly perfect, direct repeats, which also border the T-DNA in the Ti plasmid (Figure 3.2).

Ti-plasmid features

The Ti plasmid carries genes coding for plant hormones that cause cell proliferation and genes that code for proteins mediating DNA transfer.

Ti plasmids from different strains of *A. tumefaciens* generally have several features in common:

1 they contain one (or more) T-DNA regions;
2 they contain a *vir* region;
3 they contain an origin of replication;
4 they contain a region enabling conjugative transfer;
5 they contain genes for the catabolism of opines (a class of amino acid/sugar conjugates).

The T-DNA

The T-DNA region of any Ti plasmid is defined by the presence of the right- and left-border sequences (see Figure 3.2). These border sequences are 24 bp imperfect

Nopaline strains

TG(G/A)CAGGATATAT(-/T)G(T/G)(G/C)G(T/G)GTAAAC

Octopine strains

(C/T)GGCAGGATATA(T/A)C(A/C)(A/G)TTGTAA(A/T)T

TAAGTCGCTGTGTATGTTTGTTTG (Enhancer or 'overdrive' sequence)

Figure 3.2 The consensus sequences of the border sequences from nopaline- and octopine-strain Ti plasmids. The nucleotide sequence of the enhancer sequence is also shown.

repeats. Any DNA between the borders will be transferred into the genome of the host plant. Although not part of the T-DNA, octopine-strain Ti plasmids contain an overdrive sequence or enhancer sequence (see Figure 3.2) associated with the right-border sequence, which is required for optimal T-DNA transfer.

Nopaline strains of Ti plasmid have one T-DNA of approximately 20 kb, whereas octopine strains have two T-DNA regions, termed T_L and T_R, that are approximately 14 and 7 kb, respectively. However, only the T_L region is oncogenic (the T_R region carries genes for opine biosynthesis).

The T-DNA of nopaline Ti plasmids contains 13 open reading frames (ORFs), whereas the T_L T-DNA of octopine plasmids contains eight. These ORFs have the features of eukaryotic, rather than prokaryotic, genes. The nopaline T-DNA and the T_L octopine T-DNA show extensive similarity in an area known as the core region. This region contains the genes that code for proteins involved in hormone biosynthesis (the oncogenes) and opine synthesis, and for determining tumour size. The organization of the genes in the T_L T-DNA is shown in Figure 3.3.

The oncogenes

Two genes, *auxA* (or *tms*1 or *iaaM*) and *auxB* (or *tms*2 or *iaaH*), encode proteins involved in the production of the auxin indole-3-acetic acid (IAA). *auxA* encodes tryptophan monooxygenase and *aux*B encodes indole acetamidehydrolase. Another gene, *cyt* (or *tmr* or *ipt*), encodes an isopentenyl transferase that catalyses the most important step in cytokinin production. These genes are the prime determinants of tumour phenotype and are therefore often referred to as oncogenes. The T_R region of octopine T-DNA contains no oncogenes, explaining its lack of oncogenicity.

Other genes present on the T-DNA

Genes for the production of opines (either octopine or nopaline) are present on the T-DNA (the octopine T_R T-DNA possesses genes encoding proteins involved in the biosynthesis of other opines such as mannopine, agropine, and fructopine). The *tml* gene, which is involved in determining tumour size in some species, is also found in the T-DNA (see Figure 3.3).

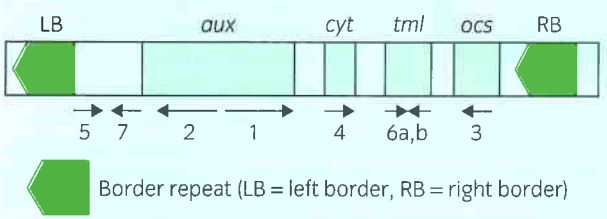


Figure 3.3 The genetic organization of the T_L T-DNA of an octopine-type Ti plasmid. Only the T_L region is shown as this has homology with the T-DNA of nopaline-type Ti plasmids. Eight ORFs are indicated (1–7), although ORFs 5 and 7 are not discussed in this text. Regions of importance are shaded light green and include the *aux* genes (which encode enzymes involved in auxin biosynthesis), *cyt*, which encodes isopentyl transferase (an enzyme involved in cytokinin production), *tml*, which is involved in regulating tumour size, and *ocs* (octopine synthase), which encodes opine synthesis. (Redrawn with permission from Hughes (1996).)

Table 3.1 *Agrobacterium* virulence protein functions

Virulence protein	Function in *Agrobacterium* spp.	Function in plant	Plant proteins that interact
VirA	Phenolic sensor kinase Part of two-component system with VirG; phosphorylates and activates VirG		
VirG	Transcription factor Responsible for induction of *vir* gene expression		
VirB1–B11	Components of membrane structure (transfer apparatus) for transfer of T-DNA		
VirC1	Overdrive-binding protein Enhances efficiency of T-DNA transfer		
VirD1	Required for T-DNA processing Modulates VirD2 activity		
VirD2	Nicks the T-DNA and directs T-DNA through the VirB/VirD4 transfer apparatus	Nuclear targeting of T-DNA Protection of 5′ end of T-DNA from nucleases Possibly involved in integration	Karyopherin-α[a] Cyclophilins[b] pp2C[c]
VirD4	Component of transfer apparatus		
VirE1	VirE2 chaperone Required for VirE2 export from *Agrobacterium* spp.		
VirE2		Single-stranded DNA-binding protein Prevents T-DNA degradation by nucleases Involved in nuclear targeting and passage through NPC	VIP1[d]
VirF		Directs proteins coating T-complex for destruction in proteasome	
VirJ	T-DNA export		

[a]Enables transport through the plant nuclear-pore complex (NPC).
[b]Cyclophilins may cause conformational changes in VirD2 or may be involved in targeting to chromatin and integration of the T-DNA.
[c]Serine/threonine protein phosphatase type 2C (pp2C) negatively affects nuclear import of the T-DNA.
[d]VIP1 facilitates nuclear import and is involved in directing the T-DNA to chromatin.

The *vir* region

The genes responsible for the transfer of the T-DNA region into the host plant are also situated on the Ti plasmid, in a region of approximately 40 kb outside the T-DNA, known as the *vir* (virulence) region.

There are at least nine *vir* gene operons (Table 3.1 summarizes *vir* gene functions). The functions of the *vir* genes present in these operons have, in many cases, been determined. In the following section, we will look in more detail at *vir* gene functions and the roles they play in T-DNA transfer from *A. tumefaciens* to plant cells.

The process of T-DNA transfer and integration

T-DNA transfer and integration into the plant genome can be divided into the following steps (see Figure 3.4 for a simplified representation of this process).

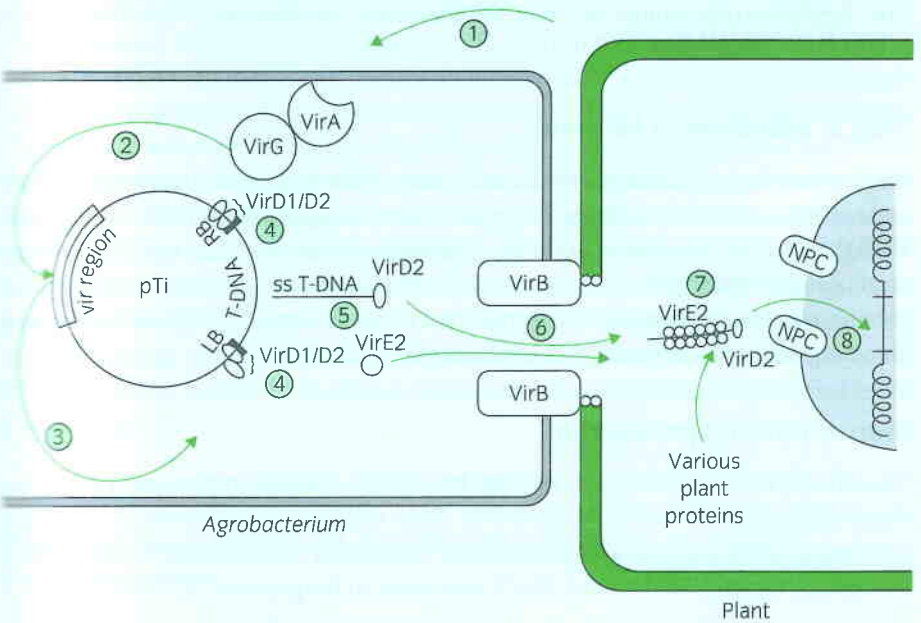


Figure 3.4 A simplified representation of the T-DNA transfer and integration process. Wounded plant cells release phenolic substances and sugars (1) that are sensed by VirA, which activates VirG by phosphorylation. VirG induces the expression of all the genes in the *vir* region of the Ti plasmid (2). Gene products of the *vir* genes (3) are involved in a variety of processes. VirD1 and VirD2 are involved in single-stranded (ss) T-DNA production, protection, and export (4 and 5). VirB products form the transfer apparatus. The single-stranded T-DNA (associated with VirD2) and VirE2 are exported through the transfer apparatus (VirF may also be exported) (6). In the plant cell, the T-DNA becomes coated with VirE2 (7). Various plant proteins interact with either VirD2 or VirE2, which are attached to the T-DNA and influence transport and integration. The T-DNA–VirD2–VirE2–plant protein complex enters the nucleus through the nuclear-pore complex (NPC). Integration into the plant chromosome (8) occurs via illegitimate recombination. LB, left border; pTi, Ti plasmid; RB, right border.

Step 1. Signal recognition by *Agrobacterium*

Agrobacterium perceives signals, such as phenolics and sugars, which are released from wounded plant cells. Normally these substances are probably part of the plant's defence mechanism, being involved in phytoallexin and lignin synthesis. The substances released from wounded cells effectively signal the presence of plant cells that are competent for transformation.

Step 2. Attachment to plant cells

Attachment of *Agrobacterium* to plant cells is a two-step process, involving an initial attachment via a polysaccharide (the product of the *attR* locus). Subsequently, a mesh of cellulose fibres is produced by the bacterium. Several chromosomal virulence genes (*chv* genes) are involved in the attachment of the bacterial cells to the plant cells. The *chvA* and *chvB* genes are involved in the production and secretion of cyclic β-1,2-glycans, and the *pscA* gene (psc, polysaccharide) is involved in secreting succinoglycan. *Agrobacterium* strains carrying mutated *chvA* or *chvB* genes are avirulent or extremely attenuated.

Step 3. Induction of *vir* genes

VirA (a membrane-linked sensor kinase) senses phenolics (such as acetosyringone) and autophosphorylates, subsequently phosphorylating and thereby activating VirG. VirG then induces expression of all the *vir* genes (including *virA* and *virG*). Many sugars, but in particular glucose, galactose, and xylose, enhance *vir* gene induction. This enhancement requires another chromosomal *vir* gene, termed *chvE*, which encodes a glucose/galactose transporter that interacts with VirA.

Step 4. T-strand production

The left and right borders are recognized by a VirD1–VirD2 complex and VirD2 produces single-stranded nicks in the DNA. After nicking, VirD2 becomes covalently attached to the 5′ end of the displaced single-stranded T-DNA strand. Repair synthesis replaces the displaced strand. VirC1 may assist in the process.

Step 5. Transfer of T-DNA out of the bacterial cell

● T-DNA is transferred to the host plant cells as a single strand of DNA covalently linked to VirD2.

The T-DNA–VirD2 complex is exported from the bacterial cell by a T-pilus (effectively a membrane-channel secretory system) composed of proteins encoded by the *virB* operon and VirD4. VirE2 and VirF are also exported from the bacterial cell.

Step 6. Transfer of the T-DNA and Vir proteins into the plant cell and nuclear localization

● Host-plant proteins are heavily implicated in nuclear localization and integration of the T-DNA.

The T-DNA–VirD2 complex and other Vir proteins cross the plant plasma membrane. Once inside the plant cytoplasm, the T-DNA strand becomes covered with VirE2 proteins and is known as a mature T-complex. The VirE2 molecules are postulated to

protect the T-DNA from nucleases, facilitate nuclear localization, and confer the correct conformation to the T-DNA–VirD2 complex for passage through the nuclear-pore complex (NPC).

Both VirD2 and VirE2 have been shown to interact with a variety of plant proteins that are important in ensuring nuclear localization of the T-DNA complex. VirD2 contains a nuclear localization signal and interacts with a plant protein. This plant protein belongs to a group of proteins (called the karyopherin-α family) involved in the recognition and transport of proteins through the NPC.

VirE2 possess two nuclear localization signals, but its nuclear localization is mediated by another plant protein, termed VirE2-interacting protein 1 (VIP1), which also has a functional homologue, the bacterial VirE3 protein. VIP1 and VirE3 are thought to facilitate interactions between VirE2 and karyopherin-α, effectively 'piggy-backing' the VirE2 into the nucleus of the plant cell. This contribution of VirE2 to the nuclear localization of T-DNA complexes may be particularly important for large T-DNAs.

The T-DNA and associated proteins pass through the nuclear pore, with the bound VirE2 proteins also giving the correct conformation to the T-DNA strand. VIP1, which interacts with the chromatin protein histone H2A, may be particularly important in targeting the T-complex to the integration site. The T-complex does, however, also interact with various proteins that form part of the transcriptional machinery and these interactions may also be important in directing the T-complex to the site of integration. The proteins bound to the T-complex must also be removed, a process involving VirF, a bacterial F-box protein that can direct VIP1 and VirE2 for degradation by the proteasomal machinery. The T-DNA strand is integrated into the host plant's genome by a process referred to as illegitimate recombination. This process, unlike homologous recombination, does not depend on extensive regions of sequence similarity and is likely to be mediated almost exclusively by plant proteins involved in DNA repair.

Practical applications of *Agrobacterium*-mediated plant transformation

We have looked at the basic biology of *A. tumefaciens* and how the T-DNA is transferred into the plant genome in some detail. We can now look at how *A. tumefaciens* is incorporated into plant transformation experiments. It is important to remember that the successful production of transgenic plants is reliant on combining a suitable transformation protocol with a robust regeneration protocol (see Chapter 2). *Agrobacterium*-mediated transformation may not prove suitable for all types of explant, and may not be the most efficient transformation method for some plant species. Nevertheless, *Agrobacterium*-mediated transformation is a widely used transformation method, partly for historical reasons and partly because of some advantages over other methods. *Agrobacterium*-mediated transformation methods are thought to induce less rearrangement of the transgene and result in a lower transgene copy number than direct DNA-delivery methods.

● *Agrobacterium* was first used to transfer DNA into dicotyledonous plant species. Subsequently it has been developed for some monocotyledon species.

CASE STUDY 3.1 *Agrobacterium*-mediated transformation of tobacco

Tobacco is a relatively easy plant to transform with *Agrobacterium* and provides a good introduction to the use of *Agrobacterium* in plant transformation. In this case study, we will look at the processes used for *Agrobacterium*-mediated plant transformation. Several factors have to be considered in the design and implementation of any plant transformation study.

1 The plant tissue to be transformed. The purpose of most plant transformation experiments in plant biotechnology is to produce whole, transgenic plants. The explant used in transformation experiments must therefore be capable of producing whole plants by regeneration, and should contain a high proportion of cells that are competent for transformation (explants and the regeneration of whole plants from them are discussed in detail in Chapter 2).

2 The vector used to deliver the transgene into the genome of the plant. The construction of vectors for use in *Agrobacterium*-mediated transformation is discussed in detail in Chapter 4. However, there are some key points, relevant to their use for plant transformation, which we can consider here (a simple vector for *Agrobacterium*-mediated plant transformation is shown in Figure 3.5). Vectors used in *Agrobacterium*-mediated transformation are derivatives of, or

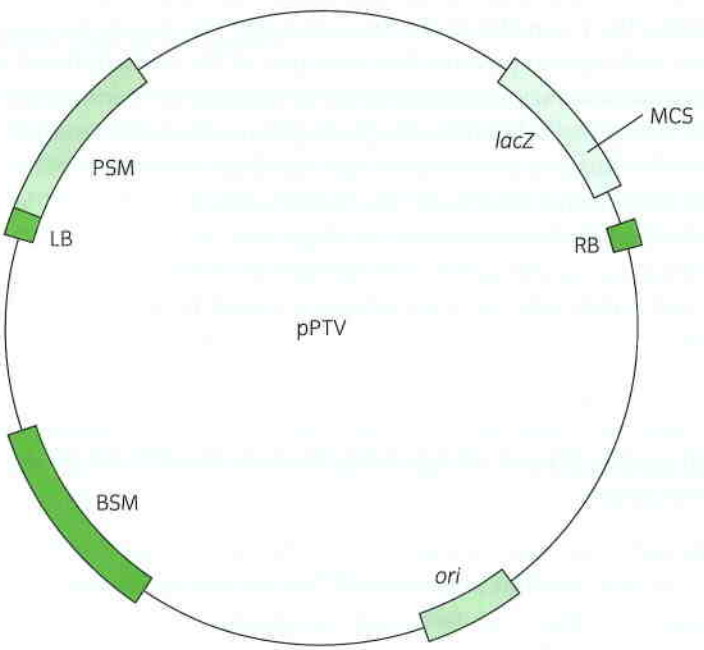


Figure 3.5 A simplified representation of a plasmid plant transformation vector (pPTV) showing the essential features of a binary-type plant transformation vector. The DNA that is transferred to the plant genome (the T-DNA) is situated between, and defined by, the left (LB) and right (RB) borders. It contains a multiple cloning site (MCS) in *lacZα* to facilitate cloning of the transgene into the vector. The T-DNA also contains a selectable marker gene to enable selection of transformed plant cells (PSM). Outside the T-DNA is a bacterial selectable marker gene (BSM) to allow selection of transformed bacteria. There is also an origin of replication (*ori*) to allow replication of the plasmid. Binary vectors like pPTV are described fully in Chapter 4.

are based on, the naturally occurring Ti plasmid. They are, however, modified extensively so that most of the features of a natural Ti plasmid are removed, only the left- and right-border sequences being used to ensure transfer of the T-DNA region between them. The vector also contains a selectable marker on the T-DNA so that transformed plants can be identified, as well as a separate selectable marker (outside the T-DNA) to enable identification of the transformed bacteria. Virulence genes required for transfer of the T-DNA are often located on a separate plasmid in the bacterium.

3 The strain of *Agrobacterium* used. Several widely used strains of *Agrobacterium* are available for plant transformation experiments. For the transformation of crops that, like tobacco, are amenable to *Agrobacterium*-mediated transformation, the choice of strain is not critical to the success of the experiment. However, for more recalcitrant plant species, the choice of strain is a major factor contributing to the success or failure of the experiment.

The basic protocol used for any *Agrobacterium*-mediated transformation experiment can be illustrated with the following example of tobacco transformation.

1 Suitable plant tissue, to be used as a source of explants, is removed from the donor plant and sterilized (if it is not from a sterile plant). For tobacco transformation, leaves are ideal.

2 The leaf tissue is cut into small pieces (using a scalpel or cork-borer) and placed into a culture of *Agrobacterium* (which contains the vector) for about 30 min, a process known as co-cultivation. During this incubation, the bacteria attach to the plant cells. The explants are subsequently removed from the bacterial culture, excess bacterial culture blotted off, and then placed on to solid Murashige and Skoog (MS) medium that contains no selective agent.

3 The incubation of the explants with *Agrobacterium* is allowed to continue for 2 days to allow transfer of the T-DNA to the plant cells.

4 The explants are removed from the medium and washed in an antibiotic solution (such as cefotaxime) that kills *Agrobacterium* cells.

5 The explants are transferred to fresh solid medium. This medium is supplemented with a selective agent (often kanamycin, but this will depend on which selectable marker gene is present in the T-DNA of the vector) to prevent the growth of non-transformed plant cells. It also contains cefotaxime (to prevent the growth of any *Agrobacterium* that were not killed by the initial treatment with cefotaxime). An auxin (1-naphthylacetic acid (NAA), 0.1 mg l^{-1}) and a cytokinin (6-benzylaminopurine (BAP), 1 mg l^{-1}) are also included to encourage regeneration by organogenesis (see Chapter 2). The relatively high cytokinin to auxin ratio promotes shoot formation from the explants. These shoots can be rooted by placing them on solid medium containing a high auxin to cytokinin ratio.

Although this is a specific example, most *Agrobacterium*-mediated transformation protocols (although see *in planta* methods described later in this chapter) follow a similar pattern, which is summarized below:

1 identify a suitable explant;
2 co-cultivate with the *Agrobacterium*;
3 kill the *Agrobacterium* with a suitable antibiotic (which does not harm the plant tissue);

4 select for transformed plant cells;

5 regenerate whole plants.

Experimental details vary depending on the plant species being transformed and the strain of *Agrobacterium* being used. There is a multitude of protocols available for the transformation of a wide range of crops, even ones such as cereals that were previously considered to be incapable of being transformed by *Agrobacterium*-mediated methods. These protocols are constantly being improved, which makes the identification of specific protocols redundant. However, some generalizations can be made.

Dicotyledonous plants, the natural target for *Agrobacterium* transformation, are, in general, easily transformed using standard vectors (see Chapter 4) and standard strains of *Agrobacterium*, such as LBA4404. Some crops, such as cereals (which are not naturally infected by *Agrobacterium*), are more difficult to transform and may require the use of modified vectors and/or so-called supervirulent strains of *Agrobacterium* (such as EHA101 or EHA105). These supervirulence systems rely on extra copies of some of the virulence genes being present either on the vector itself or on a separate plasmid in the *Agrobacterium*. Different selectable markers (Chapter 4) to identify transformed plant cells may also be required for some plant species, particularly cereals. Antibiotics such as kanamycin are commonly used to select for transformed plant cells, but alternatives such as herbicides or more potent antibiotics are often required for cereal transformation.

Transformation

Transformation protocols requiring extensive periods of tissue culture have several drawbacks, including time (typically many months), space, and, importantly, the tendency to induce somaclonal variation (i.e. the variation in plants derived from tissue culture). Hence there is considerable interest in developing transformation methods that obviate the need for tissue culture steps. Recently, such methods have been demonstrated to work in a limited number of plant species, opening up the possibility of widespread application to crop improvement. Methods for transformation *in planta* utilize either *Agrobacterium*-mediated transformation or direct gene-transfer methods (which are discussed later in this chapter). One of the most promising methods, which was developed using a member of the genus *Arabidopsis* as the target plant, is the floral dip. This is an extremely simple method in which plants with young flowers are dipped (with or without a vacuum being applied) into a culture of *Agrobacterium* that also contains a surfactant. The plant is subsequently allowed to set seed, whereupon a small proportion of the seeds produced are transgenic. Although the efficiency of this technique is very low (at present, at least), the vast number of seeds produced results in an acceptable overall transformation efficiency. This and similar techniques have been applied successfully to other plant species, including alfalfa and some brassicas.

The first part of this chapter has dealt with *Agrobacterium*-mediated transformation. However, another species of the same genus, *Agrobacterium rhizogenes*, is also capable of transferring genes to plants, and has been developed into a plant transformation system that is used in some specialized circumstances. Recently other bacterial species capable of transforming plants have also been identified and developed as plant transformation systems (Box 3.1).

BOX 3.1 Agrobacterium rhizogenes *and alternative organisms to* Agrobacterium *spp.*

Other bacterial species are now being developed for transformation.

Although *A. rhizogenes* also infects plants, it differs from *A. tumefaciens* in that the resulting pathology is not crown galls but a phenomenon known as hairy roots. At the site of infection there is a proliferation of roots. Hairy roots are important in some areas of plant biotechnology as they can be cultured *in vitro*. For many years they have been used as a source of secondary metabolites, but more recently they have been used as a system for the production of pharmaceutical proteins.

Plasmids (Ri plasmids) in strains of *A. rhizogenes* have been characterized, and it has been shown that there are a number of different types, which can be classified based upon opine usage. Hormone biosynthetic genes are also present on some Ri plasmids. Homologues of the Ti-plasmid auxin-biosynthetic genes *tms*1 and *tms*2 have been identified on the agropine-strain Ri plasmids. However, despite the well-established link between auxin and rooting, the genes are unnecessary for virulence. Rather, a series of other ORFs has been identified within the T-DNA of the plasmids, including the *rolB* and *rolC* genes, which are involved in the metabolism of plant growth regulators and which lead to the plant being sensitized to endogenous auxin. It is this increased sensitivity that leads to the root formation.

As with *A. tumefaciens*, vectors have been constructed that can be used in *A. rhizogenes* as binary systems (binary vectors are described in Chapter 4). *A. rhizogenes* transformation was, at one stage, considered an alternative strategy to *A. tumefaciens* for gene transfer as it led to the production of defined tissues (hairy roots) that could be regenerated into whole plants. This strategy seems to have been discarded, however, as more efficient *A. tumefaciens* systems have been developed.

There has been a move to develop non-*Agrobacterium*-based gene-transfer systems using other microorganisms. This is in part due to the complex landscape of patents and intellectual property rights for many biotechnological processes and in particular for *Agrobacterium*-based transformation. This issue came to a head with the development of Golden rice. This rice was to be provided free to developing countries but the legal issues took a lot of hard work to overcome (see Chapter 10). Several other issues have driven the non-*Agrobacterium* initiative: the main one being the possibility of broadening the target range of microbial-based transformations. The pathogenic nature of *Agrobacterium* has always been a problem; it can cause plant defence responses to be induced, which may lead to a reduction of transformation frequency. Another problem is that many crops are either resistant to the *Agrobacterium* infection or cannot be regenerated from susceptible tissues.

The CAMBIA group (an affiliated research centre of Charles Sturt University in Australia) have tested a number of bacterial species for their ability to transfer DNA sequences into plant tissues

(Continued overleaf)

BOX 3.1 *Continued*

so that they will be integrated, expressed, and inherited in a Mendelian fashion. Initial studies have been with *Rhizobium* sp. NGR234, *Sinorhizobium meliloti*, and *Mesorhizobium loti*. These are not plant pathogens but are either symbionts or benign endo-/epiphytes that interact with plants in different ways (in terms of target cells and growth stages), broadening the potential range of target plants. The transformation methods will need optimization but preliminary experiments have indicated significant transformation frequencies with, for instance, floral dip technologies. The aim of this work is to provide workable transformation systems with open-source licences (www.bioforge.net; Biological Innovation for Open Society, www.bios.net). These licences are without any commercial restrictions other than covenants for sharing of improvements, relevant safety information, and regulatory data, and for preserving the opportunity for others to freely improve and use the technology.

There has also been a lot of work on the development of viral systems of gene transfer; these are considered in Chapter 11.

Direct gene-transfer methods

Non-*Agrobacterium* systems are widely used for recalcitrant species.

The term direct gene transfer (or direct transfer) is used to discriminate between methods of plant transformation that rely on the use of *Agrobacterium* (indirect methods) and those that do not (direct methods). Direct gene-transfer methods all rely on the delivery of large amounts of naked DNA while the plant cell is transiently permeabilized. Several different methods or strategies for direct gene transfer have been developed over the years. Some of these, particularly biolistics or gene bombardment, have become widely adopted by plant biotechnologists.

Direct gene-transfer methods, which have found particularly widespread use in the transformation of cereal crops (which initially proved difficult to transform with *Agrobacterium*), have some advantages and disadvantages (Table 3.2) when compared

Table 3.2 Direct gene-transfer methods

Direct gene-transfer method	Comments
Particle bombardment	Very successful method. Risk of gene rearrangements and high copy number. Useful for transient expression assays.
Electroporation	Transgenic plants obtained from a range of cereal crops. Low efficiency. Requires careful optimization.
DNA uptake into protoplasts	Used for all major cereal crops. Requires optimization with a regenerable cell suspension that may not be available.
Silicon carbide fibres	Requires regenerable cell suspensions. Transgenic plants obtained from a number of species.

BOX 3.2 *Transgene integration and gene rearrangement*

The major complication found with particle-bombardment transformation methods is that the vector DNA is often rearranged and transgene copy number can be very high. Recent work on the mechanism of direct gene transfer to rice by biolistics suggests that the transfer is a two-stage process. During a pre-integration phase, vector molecules (which can be either intact or partial) are spliced together. This gives rise to fragments carrying multiple gene copies, which subsequently integrate into the host plant's genome. This integration event can act as a hot spot for further transgene integration at, or very close to, the initial point of integration. The implications of these observations are that particle bombardment generally results in the DNA being integrated in high copy number at a single locus (or a low number of loci). Despite this high copy number, the single locus may have benefits for subsequent breeding programmes.

It is not just particle-bombardment that has problems. Sequences such as the 35S promoter (Chapter 4) have been identified as recombination hot spots and some problems with *Agrobacterium*-based binary vector systems (Chapter 4) have been identified. Here, between 30 and 80% of transformants from *Agrobacterium*-based transformation have been shown to contain sequences from the plasmid outside the T-DNA region. These observations have led to a rethink in vector design for both transformation approaches. In particular, the use of all-plant-based constructs should be used now and virus or bacterial sequences should, where possible, be avoided.

● Both *Agrobacterium* and direct-transfer systems can lead to abnormal integration.

● Not transgenic but intragenic: plants transformed with 'just native plant DNA'.

with *Agrobacterium*-mediated transformation. One of the major disadvantages with direct gene-transfer methods is that they tend to lead to a higher frequency of transgene rearrangement (see Box 3.2) and a higher transgene copy number. This can lead to high frequencies of gene silencing (see Box 4.4).

The main types of direct gene transfer will be considered in some detail below. Other, less-reproducible methods, which will not be considered further, such as laser-mediated uptake of DNA, microinjection, ultrasound, and *in planta* exogenous application, have mainly been used for the analysis of transient gene expression, although stable transformation has been reported for some of these techniques on rare occasions.

Particle bombardment

Particle bombardment (biolistics) is the most important and most effective direct gene-transfer method in regular use. In this technique, tungsten or gold particles are coated with the DNA that is to be used to transform the plant tissue. The particles are propelled at high speed into the target plant material, where the DNA is released within the cell and can integrate into the genome. The delivery of DNA using this technology has allowed transient gene expression (which does not depend on integration of the transgene into the plant genome) to be widely studied, but integration

● Particle bombardment has been used widely for cereal transformation.

of the transgene occurs only infrequently. To generate transgenic plants, the plant material, the tissue culture regime, and the transformation conditions have to be optimized quite carefully.

Particle bombardment systems were first developed in 1987 and used an explosive charge to propel DNA-coated tungsten particles. This technology was the key to cereal transformation. All the major cereals were able to be transformed, and the first commercial genetically modified (GM) crops, such as maize containing the *Bacillus thuringiensis* (*Bt*) toxin gene (see Chapter 6), were produced by this method. Developments to the technology led to the production of a number of systems, such as an electrostatic discharge device, and others based on gas flow. Of the latter type, a commercially produced, helium-driven, particle-bombardment apparatus (PDS-1000/He) has become the most widely used.

Attempts to optimize the system have focused on three aspects of the process: particle type and preparation; particle acceleration; and choice of target material. A balance has to be reached between the number and size of particles fired into the target cells, the damage they do and the amount of DNA they deliver. Too little DNA may lead to low transformation frequencies, but too much DNA may lead to a high copy number and rearrangements of the transgene constructs (Box 3.2). Recently, new methods of preparing the particles for biolistic transformation, which involve the use of aminosiloxanes (instead of calcium chloride and polyamines) to coat the particles with DNA, have led to higher transformation efficiencies at lower DNA concentrations.

Plant tissues used for bombardment are generally of two types: primary explants that are bombarded and then induced to become embryogenic; or proliferating embryogenic cultures that are bombarded and then allowed to proliferate further and subsequently to regenerate. To protect the plant tissue from the damage sustained during the bombardment procedure, treatments to induce limited plasmolysis or culture on high-osmoticum media have been used.

CASE STUDY 3.2 Biolistic transformation of rice

In this case study, we will look at how the PDS-1000/He particle-bombardment system can be used to transform rice. The strategy chosen is one where two plasmids (see Figure 3.6) are introduced into the plant cell together. One plasmid (pOZ) carries the transgene of interest and the other (pHAG) carries a selectable marker (*hyg*), which confers resistance to the antibiotic hygromycin (selectable markers are described fully in Chapter 4). This plasmid also carries the *gusA* reporter gene (reporter genes are described in Chapter 4), which can be easily assayed histochemically. Both plasmids are coated on to gold particles (these particles are termed the microcarrier), for bombardment into the plant material. The plant material used for transformation is embryogenic callus derived from mature seeds (see Chapter 2).

After bombardment, cells containing the antibiotic-resistance gene are selected for on culture medium containing the antibiotic hygromycin (the selective agent). There is no direct selection for the other plasmid (pOZ), but it has been shown that in many cells both plasmids will integrate (dual transformation). In this system, then, the aim is to select and regenerate plants

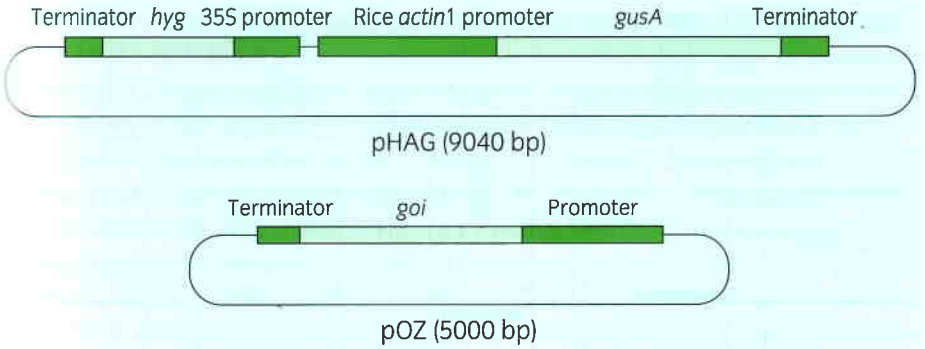


Figure 3.6 Plasmid maps of pHAG and pOZ. The plasmid pHAG contains a selectable marker gene (*hyg*), which is driven by a constitutive promoter (the 35S promoter from cauliflower mosaic virus, which is described in more detail in Chapter 4). The *hyg* gene confers resistance to the antibiotic hygromycin. pHAG also contains a reporter gene (*gusA*), which is driven by the rice *actin*1 promoter, a strong promoter in cereals. The *gusA* gene product can be easily assayed and forms a convenient way of confirming that the hygromycin-resistant plant cells are transformed and not 'escapes'. It is the presence of this plasmid that is selected for after particle bombardment by growing the putatively transformed plant tissue on medium that contains hygromycin. The plasmid pOZ is used to deliver the gene of interest, *goi*. There is no selectable marker on pOZ, and therefore the presence of this plasmid cannot be directly selected for. However, dual transformation with both plasmids is a relatively common event. The *ipt* gene is driven by a suitable promoter, the characteristics of which will depend on the required pattern of expression. The promoter may be inducible, tissue-specific, or constitutive.

in which both plasmids have stably integrated into the genome. This approach ultimately allows the selectable marker to be segregated out in subsequent generations. The resulting plants contain the gene of interest, but do not contain the hygromycin-resistance gene, which is no longer necessary. The desirability of removing antibiotic-resistance marker genes, in particular from crops, is discussed in Chapters 4 and 12.

Preparation of microcarriers

Gold or tungsten particles (beads or balls) are used as the microcarrier. These need to be pre-treated by washing the microcarriers in ethanol and sterile distilled water. Suspensions of the microcarriers can then be stored as aliquots, either at 4°C (gold) or at –20°C (tungsten). Plasmid DNA (isolated from *Escherichia coli*) is attached to the gold microcarriers by mixing the DNA and the microcarriers in 2.5 mol l^{-1} $CaCl_2$ and 0.1 mol l^{-1} spermidine (a polyamine). The microcarriers are then mixed, washed with ethanol, and finally resuspended in ethanol. The microcarriers are then applied to the macrocarrier membrane as an ethanol suspension and are allowed to dry on to the macrocarrier.

Preparation of plant material for transformation: callus induction

Dehulled, mature rice seeds are sterilized in ethanol and bleach, and subsequently washed in sterile distilled water to remove any traces of bleach. The dehulled seeds are then placed on MS1 medium (MS medium supplemented with 2.5 mg l^{-1} 2,4-D/30 g l^{-1} maltose and solidified with 2.5 g l^{-1} Phytagel; medium composition is discussed in detail in Chapter 2). The cultures are

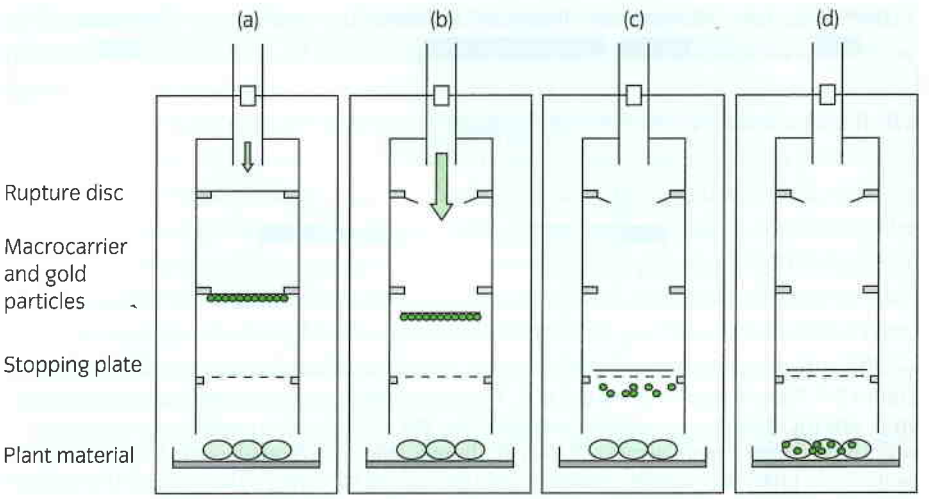


Figure 3.7 The PDS-1000/He particle-bombardment system. The plant tissue is placed into a vacuum chamber (chamber pressure 27 mmHg) 13 cm below the microcarrier stopping plate. The vector DNA-coated particles (the microcarriers) on the macrocarrier membrane are inserted into the apparatus (a). Once the vacuum in the lower part of the apparatus is established, the helium pressure above the rupture disc is increased until at 1100 psi (7584.2 kPa; or whatever pressure the rupture disc is designed to rupture at) the rupture disc bursts (b). This propels fragments of the macrocarrier and the projectiles down the chamber. The macrocarrier is stopped at the stopping plate (c), allowing the microcarriers to pass through and hit the plant material (d). Various parameters can be optimized in this system: the distance between the stopping plate and the plant material can be varied; and the pressure at which the rupture disc bursts can also be selected. Varying these parameters allows the speed and pattern of the microcarriers to be adjusted to suit the needs of the plant material being transformed.

then incubated in the dark at 25°C for 2 weeks. Callus tissue that develops from the seeds is transferred to fresh medium of the same composition and incubated for a further 2 weeks for embryogenic callus to develop. The embryogenic callus is transferred (50–70 calluses are arranged on one 9-cm diameter Petri dish) to a different medium (MS medium supplemented with 1 mg l^{-1} 2,4-D, 14% (w/v) maltose, and 2.5 g l^{-1} Phytagel) and incubated for 4 h. This medium contains a high concentration of maltose to generate a high osmoticum prior to particle bombardment of the plant tissue.

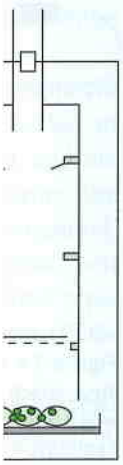
Bombardment conditions

The PDS-1000/He equipment is assembled as shown in Figure 3.7 and used to bombard the rice embryogenic callus. The operation of the PDS-1000/He equipment is also described in the figure.

Plant selection and regeneration

One day after bombardment, the embryogenic callus is transferred to MS1 medium. After 1 week, the embryogenic callus is transferred to selection medium (MS1 medium supplemented with 40 mg l^{-1} hygromycin) and incubated for 2 weeks in the dark. The callus is then transferred to fresh selection medium (of the same composition) and incubated for a further 2 weeks in the

(d)



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dark. Surviving callus is transferred to plant regeneration medium RM1 (MS medium supplemented with 3 mg l^{-1} kinetin, 30 g l^{-1} maltose, 40 mg l^{-1} hygromycin, and 6.25 g l^{-1} Phytagel) and incubated for 3 weeks in the light to encourage shoot formation. Callus is then transferred to RM2 medium (as RM1 but with 2.5 g l^{-1} Phytagel) for a further 3 weeks' incubation in the light. Green shoots that have formed on RM2 medium are rooted by transferring them on to R1 medium (half-strength MS medium supplemented with 1 mg l^{-1} NAA). Young plantlets are then subcultured on to R2 medium (half-strength MS basal medium). The plants are acclimatized in growth chambers and can then be transferred to soil and grown to maturity.

Bombardment has been used mainly for transformation of the nuclear genome, but more recently it has become the main strategy for introducing DNA into plastids (Box 3.3). Chloroplast transformation is a development for the 21st century, and its application to GM crops is discussed in detail in Chapters 11 and 12. Suffice it to say that chloroplast transformation offers opportunities for creating GM crops that are both environmentally friendly and more efficient.

BOX 3.3 *Plastid transformation*

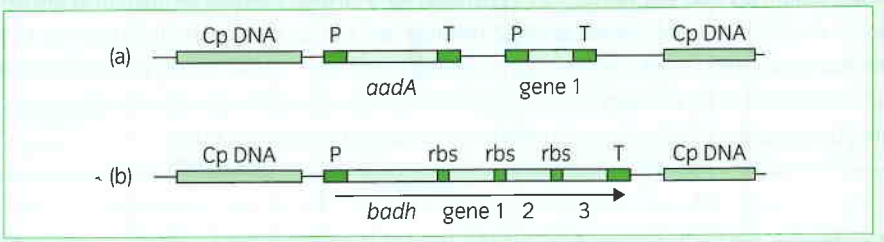
There are a number of important reasons why chloroplast transformation has become an important technique in plant biotechnology. In many, but not all, crop species, chloroplasts display only maternal inheritance so there is no danger of any gene transfer to related weedy species through pollen. Another significant feature of this technology is the very large transgene copy number that may be obtained. Transgene insertion may occur into a single genome contained within an individual chloroplast. However, by using several rounds of selection during the plant regeneration process, it is possible to develop a homoplasmic population of chloroplasts (where all the chloroplasts are transformed). As there may be as many as 100 chloroplasts per cell, each containing up to 100 copies of the genome, there may be as many as 10 000 copies of the transgene per cell. An additional advantage is the absence of reports of gene silencing with chloroplast transformation. This fact, combined with the ability of the chloroplast to correctly fold and crosslink expressed proteins, means there is a tremendous potential in chloroplast transformation for very high level gene expression and synthesis of active proteins.

This has led to the development of vectors designed specifically for chloroplast transformation. The figure shows (in panel a) a basic vector for chloroplast transformation in which selection is based on the antibiotic spectinomycin (the selectable marker gene is referred to as *aadA*). The vector is designed for the expression of a single transgene, which is fused to plastid regulatory sequences, such as the promoter from the *psbA* gene and ribosome-binding regions. The foreign genes are bordered by chloroplast DNA sequences. After introduction into the chloroplast by bombardment, homologous recombination occurs between the plastid sequences on the vector and those on the genome. This site-specific integration event avoids any problems associated with random insertion into the nuclear genome.

(Continued overleaf)

The high number of chloroplasts and the multiple copies of the chromosomes means that the cells contain many copies of the transgene, giving high levels of expression without gene silencing.

BOX 3.3 *Continued*



Vectors for chloroplast transformation. (a) A construct designed for the expression of one gene of interest (gene 1). There is also a selectable marker gene (*aadA*), which confers resistance to the antibiotic spectinomycin. Each gene (gene 1 and *aadA*) is driven by a promoter (P) and has a terminator (T). Flanking the two genes are regions of chloroplast DNA (Cp DNA) that direct integration of the DNA at a defined point in the chloroplast genome by homologous recombination. (b) A construct designed for the expression of multiple transgenes. One promoter (P) drives the expression of a polycistronic transcription unit, or operon. In this case the betaine-aldehyde dehydrogenase gene (*badh*) is used as the selectable marker. Each transgene is preceded by a ribosome-binding site (rbs) to ensure efficient translation. Other features are as in (a). (Redrawn with permission from Daniell *et al.* (2002).)

More-advanced vectors have been designed that take advantage of the prokaryotic nature of some of the plastid genome (see Chapter 1). Multiple ORFs can be introduced as a single transcription unit that is expressed as a polycistronic message (panel b in the figure). This offers a significant advantage over nuclear transformation, where individual genes are introduced separately and may have to be brought together by breeding strategies after plants with similar expression levels have been identified. A similar approach has been the development of a two-vector system, each vector carrying separate subunits that can combine in the organelle to form a functional protein. Another recombination-based approach has been to design promoterless vectors. These vectors are targeted to integrate adjacent to existing promoters.

Further development is also being carried out on the selection systems used. Strategies have been developed to remove the selectable markers, by techniques such as reversal of the homologous recombination events; some vectors incorporate the use of the betaine-aldehyde selection system, which generates much higher transformation efficiencies (see Chapters 4 and 9).

Polyethylene glycol-mediated transformation

Plant protoplasts (plant cells that have had their cell walls removed; see Chapter 2) can be transformed with naked DNA by treatment with polyethylene glycol (PEG) in the presence of divalent cations (usually calcium). The PEG and the divalent cations destabilize the plasma membrane of the plant protoplast and render it permeable to naked DNA. Once inside the protoplast the DNA enters the nucleus and integrates into the genome.

Plant protoplasts are not easy to work with, and the regeneration of fertile plants from protoplasts is problematical for some species, limiting the usefulness of the technique. The DNA used for transformation is also susceptible to degradation and rearrangement. Despite these limitations, the technique does have the advantages that protoplasts can be isolated and transformed in large numbers from a wide range of plant species.

Electroporation

● Electroporation has been used successfully to transform cereals.

The electroporation of cells can be used to deliver DNA into plant cells and protoplasts. The vectors used can be simple plasmids; the genes of interest require plant regulatory sequences, but no specific sequences are required for integration. Material is incubated in a buffer solution containing DNA and subjected to high-voltage electrical pulses. The DNA then migrates through high-voltage-induced pores in the plasma membrane and integrates into the genome. Electroporation has been successfully used to transform all the major cereals, particularly rice, wheat, and maize. Initially, protoplasts were used for transformation, but one of the advantages of the system is that both intact cells and tissues (such as callus cultures, immature embryos, and inflorescence material) can be used. This reduces some, but not all, of the tissue culture problems. However, the plant material used for electroporation may require specific treatments, such as pre- and post-electroporation incubations in buffers of high osmotic pressure. The efficiency of electroporation is also questionable: it is very dependent on the condition of the plant material used and the electroporation and tissue treatment conditions chosen.

Some studies have indicated that linear, rather than supercoiled, plasmid DNA and the addition of spermidine to the incubation buffer (which can induce condensation of the DNA) improves the efficiency of electroporation. Optimization of what is known as the field strength (the voltage applied across the electrodes) is also important.

Even under optimal conditions the amount of DNA delivered to each cell is low, which has the benefit of producing transformants with a low transgene copy number. The delivery rate (the proportion of electroporated cells that actually receive DNA) can be high: in some experiments it has been shown that between 40 and 60% of the incubated cells receive DNA, and that as many as 50% of the cells survive the treatment. Electroporation also has the advantage that all the cells are in the same physiological state after transformation, unlike the situation with particle bombardment where transformed cells may be at a disadvantage due to damage from the transformation procedure.

Silicon carbide fibres: WHISKERS™

A simple technique using silicon carbide fibres requires no specialized equipment. Plant material (such as cells in suspension culture, embryos, and embryo-derived calluses) is introduced into a buffer containing DNA and silicon carbide fibres, which is then

vortexed. The fibres, which are about 0.3–0.6 μm in diameter and 10–100 μm long, penetrate the cell wall and plasma membrane, allowing the DNA to gain access to the inside of the cell. The drawbacks of this technique relate to the availability of suitable plant material and the inherent dangers of the fibres, which require careful handling.

Although the procedure has been utilized with friable callus from maize, this type of friable callus is limited only to a few genotypes of maize and oats. Many cereals, the natural targets for the procedure, produce embryogenic callus that is hard and compact and not easily transformed, at present, with this technique. Recently though, some progress has been made in transforming such material, and procedures are being developed to allow transformation of cereals such as rice, wheat, barley, and maize without the need to initiate cell suspensions.

SUMMARY

A variety of techniques for plant transformation are available to the plant biotechnologist. These techniques can be split into two groups: *Agrobacterium*-mediated transformation and direct gene-transfer methods, of which the biolistics approach is probably the most widely used. These two groups of techniques are fundamentally different in mechanism, and are, in general, applied to different crops. *Agrobacterium*-mediated transformation is most widely used with dicotyledonous crops, which reflects the natural host range of members of the genus *Agrobacterium*. Direct gene-transfer methods are most commonly used to transform monocotyledonous crops, such as cereals. In part, this reflects the initial difficulties with using *Agrobacterium* to transform monocotyledonous plants. Direct gene-transfer methods and *Agrobacterium*-mediated methods have their own advantages and disadvantages. However, all plant transformation methods can suffer from a problem known as gene silencing (see Box 4.4), where transgene (and homologous endogenous gene) expression is actually repressed. Gene silencing is impossible to predict precisely, although some precautions in vector design and transformation protocol can be taken to reduce its frequency. Gene silencing can prove a major hurdle to the commercialization of plant transformation products.

Despite any problems, improvements in plant transformation technologies, especially when coupled with an efficient plant regeneration protocol, have seen the list of crop species that can be routinely transformed grow. Crops that were once considered impossible to transform (cereals may well fall into this category) are now transformed routinely in many laboratories around the world.

These plant transformation technologies provide the basis for the advances in plant biotechnology discussed in the remainder of this book.

FURTHER READING

Visit www.oxfordtextbooks.co.uk/orc/slaterplants2e/ for hyperlinked references that take you directly to online abstracts for the journal articles listed below:

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Web-links

Plant transformation, web-link 3.1: www.ppge.ucdavis.edu/Transformation/transform1.htm

Plant transformation, web-link 3.2: www.uoguelph.ca/~jdberg/plantran.htm

The microbial world. Biology and control of crown gall (*A. tumefaciens*), web-link 3.3: http://helios.bto.ed.ac.uk/bto/microbes/crown.htm

Transgenic crops: an introduction and resource guide, web-link 3.4: www.colostate.edu/programs/lifesciences/TransgenicCrops/how.html