10.4.1?Transformation systems

In the 1970s, several research groups attempted to introduce foreign DNA into plant genomes (Kleinhofs and Behki 1977). For example, seeds of a thiamine-requiring mutant of Arabidopsis thaliana were incubated in a solution of bacterial DNA. Plants were recovered which could grow without thiamine and it was thought that the bacterial DNA had somehow entered the plant cell and enabled the mutation to be corrected. At this time, there were no reliable methods for detecting foreign genes within plant genomes so it was difficult to prove whether the bacterial DNA had been stably incorporated. Analysis of the ‘corrected’ lines indicated the likely presence of bacterial DNA but contamination with wild-type seed could not be ruled out. Direct transformation of intact plants with naked DNA was largely forgotten for several years.

Today, however, the approach of these early workers does not seem so outlandish. We now have a greater understanding of what is required to express foreign genes in plants, coupled with several procedures to deliver foreign DNA into plant cells and methods for proving that foreign DNA has been integrated into the host genome. Gene transfer experiments are a routine tool for answering questions relating to most aspects of plant development. In addition, improvement of most major crop species by addition or deletion of specific genes is the subject of worldwide research.

Central to transferring DNA into plants is the ability to recognise individuals, whether at the cellular or whole-plant level, which have incorporated the foreign DNA (i.e. genetically transformed) against the many individuals which remain untransformed. Usually only a small percentage of cells or plants are successfully transformed in any one experiment. In the early 1980s, a new approach was modified from microbial molecular genetics to enable selection and recovery of transgenic plants. This was the introduction of antibiotic resistance genes which allow transformed cells to grow in the presence of levels of antibiotic which are toxic to wild-type parental cells. The first marker used in this way was a gene encoding neomycin phosphotransferase (NPTII) which, when fused to a promoter allowing expression in plant cells, conferred resistance to kanamycin. Alternatives include other antibiotic resistance genes such as hygromycin phosphotransferase, and genes encoding resistance to herbicides such as glyphosate or phosphinothricin.

Of course, selectable markers are only one aspect of creating stable transgenic plants. We also require systems for efficient DNA delivery and integration into the plant genome. Such methods generally fall into two categories: Agrobacterium-based transformation and physical uptake of foreign DNA.
In nature, certain soil bacteria cause disease in plants by inserting discrete pieces of DNA into the nuclear genome of plant cells. *Agrobacterium tumefaciens* infects a wide range of dicotyledonous plants via wounds and causes growth of un-differentiated callus or gall tissue. Its cousin, *A. rhizogenes*, causes outgrowths of fine roots (Figure 10.36). These species are the aetiological agents of crown gall disease and hairy root disease respectively, and can cause substantial losses in cultivated plants particularly in horticulture where infested soil remains a possible source of infection for many years.

During infection, DNA is transferred (hence the abbreviation T-DNA) from large plasmids in *A. tumefaciens* or *A. rhizogenes* into the plant genome where it is stably incorporated into plant chromosomes. The plasmid in *A. tumefaciens* is referred to as the Ti plasmid (‘tumour inducing’) and *A. rhizogenes* carries the Ri plasmid (‘root inducing’). Although the T-DNA genes in Ti plasmid and Ri plasmid differ, in both cases their collective expression in plant cells leads to a perturbation in the balance or perception of plant hormones by transformed plant cells. Galls or hairy roots can be cultured *in vitro* without the normal requirement for addition of plant hormones. T-DNA genes are eukaryotic in structure, each with its own promoter, allowing expression in plant cells. Three genes, IAAM, IAAH and IPT, cause transformed plant cells to over-produce auxin and cytokinin which leads to growth of an undifferentiated clump of cells — a gall. In *A. rhizogenes*, additional T-DNA genes collectively known as the *Rol* genes (*root* loci) may be involved in other aspects of auxin metabolism or perception, which may augment the characteristic pro-liferation of roots from transgenic cells (Costantino *et al.* 1995).

T-DNA is delineated by short border sequences of about 25 nucleotides. The right T-DNA border appears essential for transfer of T-DNA into the plant genome. DNA is nicked at this point and copied into a single-stranded form. It is then complexed with protective DNA-binding proteins and transferred from the *Agrobacterium* cell to the plant nuclear genome (Figure 10.37; Zupan and Zambryski 1995). Integration into chromosomes appears to occur at random, though transcriptionally active areas are targeted preferentially. Thus T-DNA insertion can destroy the integrity of active genes, or at least disrupt their expression. Normally this is not a problem for the transformed plant cells as, being diploid or polyploid, the other allele(s) of the mutated gene remains functional. Random integration into active genes can, however, be put to good use to help identify plant genes on the basis of altered phenotype.

Many of the genes required for DNA transfer reside on a section of the Ti or Ri plasmid known as the virulence (*Vir*) region. *Vir* genes are prokaryotic in structure and so function in bacterial cells, unlike those in T-DNA which have eukaryotic features, as noted above. Wounded plant cells produce phenolic compounds such as acetosyringone (Figure 10.37) which are detected by the protein product of one of the *Vir* genes, VirA, which then leads to the expression of other *Vir* genes. Together, the Vir proteins result in T-DNA transfer into the plant cell nucleus, probably in a manner akin to conjugative plasmid transfer between bacteria (Zupan and Zambryski 1995). Classical experiments in the 1920s by a pioneer in this field, A.J. Riker, showed that tumour formation occurred most effectively if plant tissues were incubated at 22ºC but above 28ºC there was a complete absence of tumour development. We now
know that this probably relates to T-DNA transfer which has a temperature optimum around 20ºC (Fullner and Nester 1996).

Figure 10.37 Transfer of DNA from Agrobacterium to plant depends on a complex series of interactions between host and pathogen. The process is usually initiated by compounds such as acetosyringone which are produced by wounded plant cells. T-DNA genes are expressed after incorporation into the host genome. These genes code for plant hormone biosynthetic enzymes and other genes for synthesis of unusual amino acids called opines which only the bacteria can make use of. Replacing the pathogenic T-DNA genes with genes for desirable economic traits is the basis for Agrobacterium-mediated transformation. (a) Structure of acetosyringosome (3,5-dimethoxy-4-hydroxyacephenone). (b) Schematic representation of Agrobacterium interaction and T-DNA transformation of plants (see Zupan and Zambryski 1995 for further details).

**Development of binary vectors for plant transformation**

Although wild-type T-DNA and Vir genes are located on the same plasmid, the protein products of the Vir genes act in *trans*. This means that the T-DNA may be on a different plasmid from the Vir genes in the agrobacterial cell, and still be efficiently transferred into the plant genome, provided the Vir genes are intact and functional. This has led to specialised ‘binary’ cloning vectors becoming the most commonly used Agrobacterium-based transformation systems.
In the 1980s, disarmed (i.e. non-pathogenic) *A. tumefaciens* strains were created which had the T-DNA genes deleted from their Ti plasmid so could no longer induce gall symptoms, but still retained the Vir genes and other regions essential for gene transfer into a plant host. Recombinant vectors are actually constructed in *E. coli*, and typically contain T-DNA border sequences, selectable marker genes and unique restriction enzyme sites to enable the coding sequence of any foreign gene to be ligated downstream of the chosen eukaryotic promoter (Figure 10.38). This ensures that the foreign gene is expressed in the plant genome. After inoculation with *A. tumefaciens*, cells are often manipulated in tissue culture using a high cytokinin to auxin ratio to induce shoot organogenesis with a minimum of callus formation. This assists recovery of transgenic plants lacking the genetic abnormalities which can accumulate during extended callus phases.

To date, most dicotyledonous plants of major commercial importance, as well as many species of experimental value, have been transformed using *Agrobacterium*-based transformation in conjunction with organogenesis in tissue culture. In addition, it has become possible to transform monocotyledonous grain crops such as rice, maize and barley with disarmed *A. tumefaciens*, even though it is not a pathogen on cereals (Hiei *et al*. 1994; Ishida *et al*. 1996; Tingay *et al*. 1997).

**Agrobacterium transformation without tissue culture**
Remarkable but simple transformation of *Arabidopsis* by *Agrobacterium* has been reported which does not require a tissue culture step. The procedure involves dipping inflorescences of intact plants in a bacterial suspension and forcing the bacteria into plant tissues by brief vacuum infiltration. The plants continue to grow and set seed, and a proportion of the next generation of seedlings are found to contain the inserted DNA (Bechtold *et al.* 1993).

(b) **Transformation with naked DNA**

Direct insertion of naked DNA into plant cells is an alternative transformation strategy for all species but is especially useful for plants which are less susceptible to *Agrobacterium* such as cereals and sugar cane. DNA can be from any source and again integrates into the nuclear genome largely at random. Early experiments on direct gene transfer made extensive use of plant protoplasts, with DNA uptake facilitated by treatment with polyethylene glycol or by brief exposure to an electric current (electroporation). The latter causes pores to form transiently in the plasma membrane and so allows DNA molecules to enter the cytoplasm. Protoplast culture and regeneration is now feasible for many of the world’s crops, but procedures remain relatively slow and technically demanding. Instead, DNA can be introduced into intact cells via punctures in the cell wall often in organogenic or embryogenic tissue cultures. One simple method involves vortexing cells briefly in a solution of DNA containing silicon carbide fibres. These fibres have a hardness approaching that of diamond, are less than 1 µm in diameter and puncture cells without destroying them, allowing direct entry of DNA. Stably transformed maize plants have been produced in this way (Frame *et al.* 1994).

A more popular alternative is microprojectile bombardment, often known as the ‘biolistic’ or ‘gene gun’ technique, where DNA-coated gold or tungsten particles, 1–3 µm in diameter, are literally blasted into intact tissues. The DNA diffuses off the particles and integrates into plant chromosomal DNA. Originally a gunpowder charge was used, but subsequent refinements have led to the acceleration force being provided by compressed helium or electric discharge (Songstad *et al.* 1995; Christou 1996). This is less damaging to plant cells — and less dangerous for the operators! Many crop plants, including cereals and sugar cane, have now been transformed by microprojectile bombardment. As with *Agrobacterium*-based procedures, selection for antibiotic or herbicide resistance enables recovery of the relatively small percentage of cells which are transformed from the majority which are not.
A small number of genes are used widely to optimise trans-formation procedures and to reveal spatial and temporal patterns of expression of foreign genes in transgenic plants. These genes are effective markers because their protein products are readily detectable in transgenic tissues but are absent from non-transgenic tissues. Initially, antibiotic resistance genes themselves acted as markers via suitable enzyme assays. How-ever, these have largely been superseded by more sophisticated markers whose protein products can be located histochemically by simple staining techniques. The \textit{uidA} gene from \textit{E. coli} encodes the enzyme b-glucuronidase, usually referred to as the GUS gene or enzyme, which hydrolyses substrates such as methyl umbeliferyl glucuronide (MUG) to produce methyl umbeliferone (MU). When illuminated at 360 nm, MU fluoresces strongly at 455 nm, allowing levels of active GUS protein to be measured (Jefferson \textit{et al.} 1987). Alternatively, GUS can be visualised by staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-b-D-glucuronide, also known as ‘X-gluc’. Hydrolysis results in an insoluble indigo blue dye which marks cells expressing the gene (more accurately, those containing the GUS protein) (Figure 10.39a). GUS activity is generally regarded as a good indication of the strength or tissue-specificity of expression of the promoter to which the GUS gene is fused, although this is not universally so (Taylor 1997).
Other reporter systems include genes encoding luciferase, from the ?refly Pho?nis pyralis or the luminescent bacterium Vibrio. The protein product is detected in transgenic plants after addition of luciferin substrate which, in the presence of O\textsubscript{2} and ATP, results in emission of green-yellow light. A liquid-nitrogen-cooled luminometer with a sensitivity capable of detecting individual photons allows quantification of emitted light and results in spectacular pictures (Figure 10.39b).

Most reporter genes have the disadvantage that a chemical substrate must be infiltrated into the tissues and often this is toxic to plant cells. In addition, non-uniform distribution of substrate may distort the apparent location of reporter gene expression. One alternative is a protein with inherent detection properties, such as green fluorescent protein (GFP) from the jelly?sh Aequorea victoria. GFP fluorescence is non-toxic and does not require addition of substrates or cofactors (Figure 10.39c). Non-invasive detection allows monitoring of GFP gene expression in the same transgenic plant throughout its life cycle. Modification of the DNA sequence of the native jelly?sh gene has resulted in GFP genes which function even more effectively in plants, enabling detection in intact plants containing GFP at only about 0.1% of total leaf protein (Haseloff et al. 1997; Leffel et al. 1997).

(d) What factors affect expression of foreign genes?

Following transformation, we need to establish whether foreign genes remain intact in the host genome, and that they are expressed at desired levels in target tissues, and in response only to expected chemical or environmental stimuli. The foreign gene’s local environment within the genome can affect its expression. We therefore often find great variation in levels of protein or in phenotypic effects between different individuals even when transformed with the same gene. This is usually referred to as ‘position effect’. Transgene inactivation, sometimes due to methylation of DNA, can also complicate assessment of foreign gene expression. In co-suppression, the incoming gene may lead to inactivation or reduction in expression of homologous endogenous genes (Finnegan and McElroy 1994; Matkze and Matkze 1995). Transformants with desired levels of gene expression can be selected from parallel screening of several transformed lines. Those which contain only one copy of the foreign gene generally show reproducible patterns of expression. In these, the transgene will segregate in a simple Mendelian manner (1:2:1 for homozygous transgenic:hemizygous (1 copy):homozygous non-transgenic).

The largely random nature of T-DNA and naked DNA integration into higher plant genomes is usually regarded as being due to illegitimate recombination. Lower plants and fungi, on the other hand, will often integrate foreign DNA by homologous recombination if the incoming DNA is flanked by specific host DNA sequences. Similar targeted integration into pre-selected sites in higher plant genomes could reduce position effect variability of transgene expression. Homologous recombination does occur in plants, but at a frequency several orders of magnitude lower than illegitimate recombination. This may be because plants have efficient somatic DNA repair mechanisms which help them survive in natural environments where they are exposed to ultraviolet and other DNA-damaging agents. The challenges now are to improve rates of homologous recombination, and to devise methods to distinguish transformants in which homologous recombination has occurred from the large number with random insertion. Homologous recombination may then be harnessed for targeted delivery of foreign genes into precise locations within plant genomes.

One area where intentional reduction of endogenous gene activity has found many applications, in both fundamental and applied research, is the use of ‘antisense’ technology and related methods (Bourque 1995). Although the exact mechanism of antisense action remains unclear, transcription of genes in an antisense orientation often reduces expression of complementary (sense) host genes. In general, antisense sequences require high homology with endogenous genes for effective suppression of expression, and are usually driven by a strong promoter (e.g. CaMV35S) to provide sufficient antisense RNA to ‘mop up’ endogenous sense transcripts. Endogenous mRNA levels are usually reduced when
antisense sequences are present. The simplest explanation is that antisense mRNA forms a double-stranded RNA molecule with the endogenous RNA sense strand, so preventing it from functioning in translation, with the double-stranded RNA being rapidly degraded. In summary, gene sequences from any source can now be inserted into plant nuclear genomes. Techniques usually lead to random gene integration into transcriptionally active parts of the genome. Antisense expression can diminish, or almost eliminate, expression of endogenous genes. In future, it may be possible to use targeted instead of random insertion, which should lead to more predictable levels of transgene expression and should also allow deliberate disruption of specific genes to study effects on plant growth and development. In the next section, we examine expression of novel genes in plants and altered expression of resident genes. Only 15–20 years ago such experiments were in the realms of the imagination.

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