Chapter 1
Plant Nuclear Transformation

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1.1 Introduction to Plant Transformation

"Transformation" is most simply defined as a "change". In the plant biotechnology community, transformation can be a little more precisely defined as the process of DNA introduction into a plant cell, leading to a permanent change in the genetic makeup of the target cell and its derivatives.

The ability to produce whole plants from transformed plant cells, first reported by Horsch et al. (1985), has revolutionized the plant sciences and changed the face of the planet, through the success and rapid adoption of genetically modified crops. Although the transformation process itself was initially limiting, all crops of major interest have been successfully transformed and many if not most transformation technologies are considered routine. Some crops do remain a little recalcitrant to transformation and improvements in the methods for production of stably-transformed plants are still needed. The current limitations in the production of transgenic plants for both basic research and commercial application include more efficient production of transformed plants and obtaining more predictable insertion and expression of the introduced DNA.

1.1.1 DNA Introduction Basics

DNA introduction can impact and modify any of the organelles within the plant cell that also contain DNA. Suitable targets include the nucleus, plastid and mitochondrion. Plastid transformation is presented in the next part of this chapter while this

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portion of the chapter focuses exclusively on nuclear transformation. Transformation of the mitochondrion has been reported for some organisms (Johnston et al. 1998) but has not yet been reported for higher plants.

For (nuclear) transformation to be successful, DNA must first be introduced into the target cell. The DNA molecule is sufficiently large so that a physical entry point through the cell wall and cell membrane must be established and this can compromise the health of the targeted cell. After passage through the plant cell wall and membrane, the introduced DNA must then proceed to the nucleus, pass through the nuclear membrane and become integrated into the genome. It is believed that the introduced DNA can function for a short time in the nucleus as an extrachromosomal entity, but integration into the genetic material of the target cell is necessary for long-term functionality and expression.

To recover a transgenic plant, the single cell that is the recipient for DNA introduction must be capable of either forming a whole plant or contributing to the zygote, through either the pollen or the egg. Therefore, successful recovery of transgenic plants largely relies on the ability to either transform the pollen/egg directly (Ye et al. 1999) or the non-gametic cells (somatic cells; Horsch et al. 1985), which must be subsequently manipulated to form whole plants. In most cases, plant transformation relies heavily on the ability of the plant cells and tissues to form whole plants through the tissue culture process. Efficiencies, or at least the ease of transformation, would be tremendously increased if regeneration processes were improved. As things now stand, the methodologies for transformation that are described in this book are consistent and workable but improvements are always desirable.

1.2 Transient Expression

Transient expression is exactly what the phrase suggests: a short-term expression of the introduced DNA(s). Directly following the introduction of DNA into the nucleus, that DNA starts to function. Transient expression is usually studied using scorable marker genes, which report their expression via direct production of a detectable colored/fluorescent compound or an enzyme that can convert a non-pigmented substrate into a pigmented form. The most commonly used scorable markers are β-glucuronidase (GUS) from *Escherichia coli* which converts a colorless substrate into a blue form (Jefferson 1987) and the green fluorescent protein (GFP) (Chalfie et al. 1994) which fluoresces green upon excitation by high-intensity UV or blue light. Although expression from the *gus* gene is relatively simple and inexpensive to detect, the GUS assay itself is toxic and can therefore only be used for single time-point expression assays. In contrast, proper analysis of expression of the *gfp* gene requires costly instrumentation but gene expression can be continually observed in the target tissues over time.

Transient expression represents the first indication of successful gene introduction and function. In the development of new DNA introduction methodologies,

observation of a single blue GUS-expressing cell or a few GFP spots is usually all that is needed to suggest further investigations of an approach. Transient expression in a cell should be very clear following visualization of GUS or GFP. Expression is most often limited to the targeted cells and a demarcation of expressing and non-expressing areas should be apparent. Faint or diffuse expression of the marker genes (if regulated by the appropriate promoter) is usually an indication of improper assay conditions.

Most transformation procedures were developed based on optimization of DNA delivery using transient expression analyses. Transient expression for GUS is typically observed 24 h post-introduction (Klein et al. 1988) while GFP expression can be observed as early as 1.5 h after delivery (but peak expression usually occurs at 8–24 h; Ponappa et al. 1999). Although studies of transient expression itself are not common, these studies do provide information on the early fate of the introduced DNA.

1.2.1 Optimization of Transient Expression

Since transient expression is a direct measure of successful DNA introduction and function, development of methods to improve transient expression has often been used as a means of optimizing the transformation process itself (Klein et al. 1988). This approach has been quite useful and successful over the years. However, transient expression is only a measure of successful short-term transgene expression and it may not always perfectly reflect the ability of the cells to integrate the introduced DNA to generate stable events. As stated earlier, as the DNA molecule is so large, the process of DNA introduction itself requires that the integrity of the cell be compromised in some way. Target tissues and cells can therefore be sufficiently damaged by the DNA delivery process so that they express the transgene at high levels but not survive over the long term. This point of diminishing returns cannot be precisely defined for the different systems but it does exist. Optimization of transient expression is quite useful for the initial development of transformation methods but the efficiency of stable transformation and stability of transgene expression should be the ultimate goals of most transformation efforts.

1.2.2 Transient Expression to Study Gene Expression and Stability

In addition to using transient expression to optimize transformation and DNA introduction methods, this type of rapid transgene expression can also be used to facilitate speedy analysis of factors that influence the strength and stability of transgene expression (Sheen 2001; Dhillon et al. 2009). Once transient expression is optimized and standardized for a specific target tissue, the effects of factors that influence the level and profile of transgene expression can be reliably determined.

Quantification of transient expression, required for this type of analysis, involves either the extraction of the gene product from the targeted tissues (Klein et al. 1987) or the use of image analysis for continual monitoring of *gfp* gene expression over time (Finer et al. 2006). Tracking of GFP expression coupled with image analysis has tremendous advantages over tissue extraction as gene expression in the same piece of tissue can be followed over time.

Transient expression analysis has been utilized to study the relative strengths of different promoters and promoter fragments (Chiera et al. 2007) and to evaluate genes that modulate the introduced transgene via gene silencing (Chiera et al. 2008). Surprisingly, promoter analysis using transient expression does not appear to reflect promoter tissue-specificity (Finer, unpublished data), which suggests that large amounts of pre-integrative DNAs do not behave exactly like single- or low-copy integrated genes. However direct promoter strength comparisons do appear to be transferable from transient expression studies to expression in stably transformed tissues (Hernandez-Garcia et al. 2009). Promoter isolation and evaluation could increase tremendously with the increased availability of genome sequences from a number of different plants. Since the production of stably transformed plants can take from weeks to months, the use of transient expression may be desirable when rapid promoter analysis is needed.

Transient expression has been used to evaluate factors that influence the stability or consistency of gene expression (Dhillon et al. 2009). As gene expression variability among different events is a significant limitation in the production of transgenics, this approach may be quite useful as a preliminary evaluation tool for transgene stabilization work. The final determination of factors that modulate transgene expression must ultimately be made only following introduction to plant cells for stable transformation.

1.3 Agrobacterium Background

Agrobacterium tumefaciens is a soil-borne bacterium that causes crown gall disease in plants. Infected plants display a gall on the stem which is composed of proliferating plant cells that were transformed with bacterial DNA. The wild-type bacterial pathogen has the special ability to invade accessible areas of the target plant, adhere to certain types of plant cells and insert some of its own DNA (Bevan and Chilton 1982). This DNA is coated with different bacterial encoded proteins, which protect the DNA from degradation, direct transport to the nucleus and assist with the integration of bacterial DNA into the plant genomic DNA. The bacterial DNA that is transferred (T-DNA) is located in the bacterial cell on a native plasmid, called the tumor-inducing plasmid (Ti plasmid). In the wild-type bacterium, the T-DNA contains genes for synthesis of nitrogen-rich opines (which are metabolized by associated bacteria) and plant hormones, which cause rapid cell proliferation leading to the formation of galls.

This brief background on *Agrobacterium* is significant as the current era of plant biotechnology was born after Mary-Dell Chilton (Bevan and Chilton 1982) and Jeff Schell (Zambryski et al. 1983), along with scientists at Monsanto (Horsch et al. 1985), found that they could replace the native opine- and hormone-producing genes in the T-DNA with any gene(s) of interest and introduce those genes into plant cells. With the opine- and hormone-producing genes removed, the T-DNA becomes "disarmed". A large number of additional discoveries enabled *Agrobacterium* to become the transformation vehicle of choice for many if not most plant transformation systems.

1.3.1 A String of Improvements for Agrobacterium

The use of *Agrobacterium*, in its original form, for the transformation of plant cells was both inefficient and unwieldy. First, the Ti plasmid was difficult to manipulate for introduction of genes of interest as it was so large. In addition, the bacterium was originally only able to infect and transform a limited number of plants and even specific cells within those plants. Due to perceived host-range limitations, grasses and monocots in general were thought to be unresponsive to *Agrobacterium*-mediated transformation. Last, wounding of the target tissue was deemed absolutely necessary as an entry point for the bacteria.

To make DNA introductions and manipulations simpler, binary vector systems were developed for use with *Agrobacterium* (DeFramond et al. 1983; Bevan 1984). The wild-type Ti plasmid contains both the T-DNA and a virulence (vir) region that encodes for genes involved in the T-DNA transfer machinery. Binary vectors allow for the separation of function on different plasmids; the Ti plasmid retains the vir region (T-DNA is removed) and the modified T-DNA is placed on the smaller binary vector, which can be more easily manipulated in the laboratory. The *vir* genes act *in trans*, leading to the processing of the T-DNA from the binary vector, for delivery to the targeted plant cells.

The host range limitations, originally associated with this biological pathogen and vector, have been largely overcome. As with most pathogens, different pathovars exist, which show different infectivity on different plants and cultivars of plants. Various *Agrobacterium* strains, which were selected for their high virulence, are now routinely used for plant transformation. The single advance, which had the greatest impact on increasing the host range for *Agrobacterium*, was the discovery that wounded plant tissues produced acetosyringone (Stachel et al. 1985), which subsequently induced some of the *vir* genes to initiate the T-DNA transfer process. Acetosyringone is now routinely included in the plant/bacterial co-culture medium at 100–200 μM. This chemical inducer of T-DNA transfer shows no deleterious effects on plant growth and development and it is always best to include this compound during co-culture, rather than risk the chance of obtaining inefficient transformation. As an alternative to including acetosyringone, *Agrobacterium* has been generated which constitutively expresses the *vir* genes (Hansen et al. 1994), which can give similar results.

1.3.2 *Agrobacterium*– Plant Interactions

The molecular mechanisms for the T-DNA transfer process have been described in detail in numerous excellent review articles (Zambryski 1992; Tzfira and Citovsky 2006) and are not presented again here. But, in order to better appreciate transformation methods that are *Agrobacterium*-based, it is best to have a basic understanding of the interaction of the bacterium with the target plant cells.

In order to transfer its T-DNA to the plant cell, the bacteria must obviously be in very close proximity to the target cell. It is well established that *Agrobacterium* binds to the plant cell and forms a pilus, which is the conduit for the transfer of DNA. It is also widely recognized that bacterial infection is mediated in most cases through wounding of the plant tissue. Wounding serves two different functions; it leads to the release of acetosyringone (in many plants) and allows the bacterium access to many different tissues. Simple preparation of the explant for culture is normally sufficient for wounding (Horsch et al. 1985) but additional wounding of some tissues with a scalpel blade is often helpful (Hinchee et al. 1988). More controlled wounding can lead to even higher transformation rates through the production of large numbers of small entry points for the bacteria (Bidney et al. 1992; Trick and Finer 1997).

Once the bacteria "enter" wounded plant tissue, it is not exactly clear where they go. In some cases, the bacteria enter the intercellular space that exists within most plant tissues and simply bind to the outside of the cell (Ye et al. 1999; Vaucheret 1994). In certain cases where the cell wall has been stripped from a plant cell, the bacteria bind to the outside of the regenerating cell wall (Deblaere et al. 1985). In the majority of cases, where the target tissue is wounded, it remains unclear whether the bacteria migrate to the intercellular spaces between cells, adhere to portions of torn/wounded cell walls, or actually colonize wounded plant cells to transform adjacent living cells (Trick and Finer 1997). Since the ultimate goal of transformation scientists and plant biotechnologists is to produce transgenic plants, the precise location of the bacteria during the transformation process is not really a nagging question. However, it is often helpful to visualize bacterial binding and the transformation process itself, when working to produce transgenic plants.

1.3.3 Reducing Agents

Although the *Agrobacterium* strains that are in common use for transformation have been engineered to achieve enhanced transformation rates, they are still perceived by many plant tissues as a pathogen. In response to pathogen invasion, plant tissues display high peroxidase activity to inhibit the growth of the pathogen and initiate localized plant cell death, so that the pathogen cannot spread through dying plant cells. During transformation with *Agrobacterium,* pathogen infection is actually desirable and inclusion of reducing agents can be used to alleviate the effects

of oxidizing agents and cell death (Olhoft et al. 2001; Finer and Larkin 2008). Reducing agents such as ascorbate, cysteine, silver nitrate and dithiothreitol have been successfully used to minimize the effects of oxidizing agents and to improve transformation efficiency.

1.3.4 Agroinfiltration

If there were a model plant family for *Agrobacterium*-mediated transformation and transgenic plant regeneration, it would be the Solanaceae. *Arabidopsis* is a special case and is presented in the next section. For the production of stably transformed plants, *Nicotiana tabacum* was often used in the early years of transformation as it is quite susceptible to *Agrobacterium* and it can be easily regenerated from almost all types of tissue (Bevan 1984; Deblaere et al. 1985). In some cases, the production of whole transgenic organisms may not be needed if a large number of cells within a plant can be uniformly and consistently transformed. During agroinfiltration (Vaucheret 1994), an *Agrobacterium* suspension is injected or infiltrated into leaves of *N. benthamiana*. The bacteria enter the intercellular air spaces within the leaf and transform a very large percentage of the internal mesophyll cells. The bacteria can be introduced into the internal leaf spaces by active pushing using an *Agrobacterium*-loaded syringe or by dipping the plant in an *Agrobacterium* suspension and then applying a vacuum. Agroinfiltration can give rise to very high levels of transgene expression in leaves of infiltrated plants when the T-DNA is modified to contain viral gene components to launch the viral amplification and transfer machinery (Lindbo 2007). This method can be used to rapidly generate a chimeric plant, where a large number of leaf cells contain the gene of interest. Unfortunately, this approach is not widely applied to different plants and is even limited among *Nicotiana* species. Inheritance of the transgene in agroinfiltrated plants does not occur.

1.3.5 Arabidopsis Floral Dip

Due to the small size of the genome and ease of transformation, *Arabidopsis* continues to serve as the model for plant genomics. The *Arabidopsis* floral dip method is a unique transformation method among plants. It was developed specifically for *Arabidopsis* (Clough and Bent 1998) and it has been shown to consistently work with very few other plants (Lu and Kang 2008). During floral dip, the *Arabidopsis* plant is first submerged in an *Agrobacterium* suspension, similar to one form of agroinfiltration (above). Inclusion of the wetting agent Silwet in the suspension and the application of vacuum, encourage the uptake of *Agrobacterium* by the plant. As the plant grows, the bacteria co-exist within the plant, eventually transforming the unfertilized egg within the ovule (Desfeux et al. 2000).

During the co-culture period, the *Agrobacterium* appear to proliferate at low levels within the plant. Plant infection does not lead to plant death nor does the plant invoke the hypersensitive response to limit the spread of the bacteria. The infecting *Agrobacterium* could transform leaf, petiole and other somatic cells of the plant but these transformation events are not passed onto the subsequent generation and are of limited value. The real benefit of the *Arabidopsis* floral dip is the rapid production of transgenic seed without the need to use tissue culture and in vitro regeneration from a single cell. The method is ideal, because the single cell that is targeted for transformation (the egg) is already destined to become a whole plant.

Since *Arabidopsis* can rapidly produce a large number of seeds and the plants are so small, space requirements are minimal and any inefficiencies in transformation is compensated by the ability to screen large numbers of seeds/seedlings. Seeds are simply plated on selective media, or seedlings/plants can be screened for certain characteristics or phenotypes to recover whole transgenic plants. Each transgenic seed usually represents an independent transformation event.

The inability to apply the *Arabidopsis* floral dip method to most other plants is not from lack of effort. In fact, successes using the same general approach with other plants have been reported but almost all of these have not been confirmed or repeated. It remains unclear why this method has not been widely applied to all plants. The transformation community remains cautiously optimistic that this approach will eventually be utilized for the transformation of all plants.

1.4 Particle Bombardment

Although *Agrobacterium* has become the method of choice for the transformation of plants, most of the first commercialized transgenic plants were generated using particle bombardment (Koziel et al. 1993; Padgett et al. 1995). Particle bombardment is a physical method for DNA delivery and the complexities of biological incompatibilities that are frequently encountered with *Agrobacterium* are completely avoided. This is also a direct DNA introduction method and it is therefore not necessary to use *Agrobacterium*-based binary vectors. DNA can be introduced as intact plasmids, isolated fragments, or PCR-generated amplicons. However, binary vectors containing genes of interest can also be used. With direct DNA introduction, DNA in any form can be utilized.

During particle bombardment, DNA is initially precipitated on small dense particles, usually 0.6–1.0 μm tungsten or gold. The particles are accelerated at high speed towards the target plant tissue and penetrate through the cell wall to eventually lodge adjacent to, or directly in the nucleus (Yamashita et al. 1991; Hunold et al. 1994). The DNA, which was initially precipitated onto the particles, is released into the cell, finds its way to the nuclear DNA and becomes integrated into the genome.

1.4.1 Gene Guns

Particle bombardment does require appropriate instrumentation to propel the particles towards the target tissue. This instrumentation should provide a means to direct the DNA-coated particles, hold the target tissues in place for particle delivery and offer a mechanism for directing and controlling the force needed to accelerate the particles. With the original gene gun, that accelerative force was generated from a 0.22 caliber powder load (Klein et al. 1987) and the devices that are used today for particle bombardment are fittingly called "gene guns". Numerous gene gun designs have been published but the two main versions in use today are the commercially available BioRad PDS1000He and the particle inflow gun (PIG; Finer et al. 1992).

The BioRad device utilizes very high-pressure helium to accelerate a lightweight mylar disc, which is layered on one side with DNA-coated gold particles. The mylar disc (macrocarrier) is accelerated into a stopping screen, which retains the mylar disc but allows the particles to pass. The PIG utilizes low-pressure helium to accelerate DNA-coated tungsten particles directly in a stream of helium. Helium is used in both cases because it is inert and its expansion coefficient is high, which means that the compressed helium gas is accelerated rapidly into a vacuum. A vacuum is not absolutely required but use of a vacuum chamber for particle bombardment is beneficial, as air drag on the accelerating particles is reduced.

1.4.2 Optimization of DNA Delivery

Particle bombardment, as with many of the DNA introduction methods, is rough on the target cells, as the integrity of the cell must be compromised to introduce the large DNA molecule. To get a more accurate picture of the scale of participants, the particles that enter the cell are in the range 0.6–1.0 μm while the target plant cells are usually 20–30 μm. If multiple particles or clumps of particles enter the same cell, damage to the target tissue increases. Ultimately, transient expression studies can be used to gauge the success of DNA introduction; living cells display transient expression while dead or severely damaged cells do not. It is unclear how many cells are moderately damaged and express transiently prior to cell death. For the optimization of DNA delivery through transient expression analysis, parameters that are evaluated usually include the following: DNA concentration, helium pressure, distance from point of particle acceleration to target tissue, DNA precipitation conditions and particle size.

The damage to the target tissue can be partly overcome through either chemical or physical drying, resulting in plasmolysis of the cells (Vain et al. 1993). Plant cells, which are normally hypertonic, push their cytoplasm through any large gaps in the cell wall. But plasmolyzed cells retain their cytoplasm following bombardment, resulting in higher transient expression and stable transformation.

1.4.3 Control of DNA Integration Patterns

One of the most interesting outcomes from particle bombardment-mediated transformation is the DNA integration pattern that can result from the introduction of plasmid DNA. Although the introduction of cassettes or amplicons is preferred over intact plasmids, the DNA integration patterns resulting from the introduction of whole plasmids has provided valuable information on the mechanism of integration following direct DNA uptake.

If intact plasmids are used for particle bombardment or any of the other methods of direct DNA deliver (see later in this chapter), the DNA integration pattern can be quite complex. Integration patterns show that plasmids can mix via both homologous and illegitimate recombination, resulting in the integration of high copy numbers of full-length plasmids, as well as pieces and parts (Finer and McMullen 1991). In addition, although the introduced DNAs segregate as a unit and are physically linked, introduced DNAs are often interspersed with plant DNA (Pawlowski and Somers 1998). One can envision the integration of introduced DNA into the plant genome using the native DNA replication and repair machinery working with the DNAs that are locally available (mixing of introduced DNAs and native genomic DNA). If desired, large amounts of DNA can be introduced (Hadi et al. 1996) and the co-introduction of two or more different pieces of DNA can be extremely efficient, leading to co-integration.

Introduction of either large amounts of different DNAs or high copy numbers of the same gene are generally undesirable as it leads to gene silencing. The use of low concentrations of isolated cassettes, generated via PCR, yields more predictable gene integration and transgene expression patterns (Agrawal et al. 2005). Apparently, the use of fragments for DNA introduction minimizes homologous recombination and concatemer formation, and the concentration of DNA used for bombardment can be reduced significantly, without reducing the recovery of transgenic events.

1.5 Other Direct DNA Uptake Approaches

The most commonly used method for direct DNA uptake (or naked DNA introduction) is particle bombardment. However, other methods have also been developed which are based on the same principle of passing DNA though large pores or holes in the cell wall or membrane. Some of these methods are very efficient in the introduction of DNA but inefficient for the recovery of transgenic plants. Other methods may not be very amenable for DNA introduction but generation of plants from the target tissue is more straightforward. These methods were developed either for purely scientific reasons, for unique applications, or to avoid the intellectual property restrictions of current DNA introduction methodologies.

1.5.1 Protoplasts

Protoplasts are plant cells with their cell wall removed. Since the cell wall presents the most formidable barrier to the introduction of large molecules, removal of the cell wall increases the possibilities for DNA insertion. For cell wall removal, tissues are incubated with commercial mixes of cellulases and pectinases. During protoplast liberation, protoplasts are suspended in a salt solution containing sufficient amounts of osmotic stabilizers to prevent bursting (Cocking 1972). Protoplasts can be prepared using any starting material but the selection of tissues depends on the desired outcome of the experiments. Protoplasts have been very successfully used in transient expression studies for fast analysis components that influence gene expression (Sheen 2001). For transient expression studies, leaf tissues as well as rapidly proliferating non-regenerable suspension cultures are suitable for the isolation of protoplasts. If transgenic plant recovery is desired, embryogenic suspension cultures are the preferred starting material.

Although protoplasts are devoid of their cell wall, the introduction of DNA molecules into these cells still requires that the DNA crosses the membrane. The two main methods for passing DNA through the membrane of plant protoplasts are electroporation (Fromm et al. 1985) and polyethylene glycol (PEG) treatment (Lazzeri et al. 1991). Both methods lead to temporary membrane destabilization, resulting in pore formation, which allows the DNA to pass. For electroporation, an electric charge is applied to the protoplasts, while the PEG treatment involves gradual application and subsequent dilution of a concentrated PEG solution to a protoplast/DNA suspension.

DNA introduction into plant protoplasts is relatively straightforward and efficient. Because the procedures for direct DNA uptake into protoplasts can be harsh, protoplast survival is a concern but roughly half of the surviving cells take up the foreign DNA. In spite of the difficulties associated with plant recovery from protoplasts, protoplast transformation remains a useful tool for transient expression studies. Since plant recovery from protoplasts is so technically demanding, this procedure is not often used for the recovery of transgenic plants.

1.5.2 Whole Tissue Electroporation

To avoid the technical difficulties encountered with the manipulation of protoplasts, the introduction of DNA through electroporation of whole tissues has been explored. Attempts to electroporate DNA into completely untreated target tissues have not been reliable. Although seemingly positive results have occasionally been obtained, these have not been consistent. Whole-tissue electroporation is achievable following partial digestion or removal of cell wall material (D'Halluin et al. 1992) using a nominal enzyme treatment. With a reduced or eliminated cell wall, the membrane is exposed and osmotic stabilizers are needed to prevent cell rupture.

Electroporation of treated tissues in the presence of naked DNA causes pore formation and results in the uptake of DNA by the plant cells. Although this approach would seem to offer many advantages over protoplast transformation in the ease of plant recovery from more "intact" tissue, very few valid reports of whole tissue electroporation exist in the literature (D'Halluin et al. 1992).

1.5.3 Silicon Carbide Whiskers

Silicon carbide whiskers are long thin rigid microscopic rods (1 μm wide, 20–30 μm long) that are used in the ceramics industry. They can be used as a vehicle for plant cell transformation when they are added to a mixture of plant cells and DNA and subsequently shaken at high speed (Kaeppler et al. 1990). Although silicon carbide whiskers were originally used with a laboratory vortexer, the back-and-forth motion obtained with a paint can mixer may work as well or better. The basic concept behind this method is to penetrate the plant cell wall with the whiskers, which carry DNA along into the cell. It appears that this penetration occurs as a result of a rod being lodged between cell clusters when they collide during the mixing. An alternate suggestion, that the silicon carbide whiskers act like flying spears to penetrate the cell wall, seems less likely as the mass of the rods is so low. This method has been successfully and consistently used but the mixing treatment is fairly harsh and the target tissues are limited to cell cultures.

1.5.4 Nanofiber Arrays

The use of nanofiber arrays for DNA introduction into plants is a relatively new approach for DNA introduction into plant cells and few reports of this method exist (Finer and Dhillon 2008). Nanofiber arrays are precisely arrayed thin fibers, which are grown directly on a silica chip (Melechko et al. 2005). When viewed using electron microscopy, these chips resemble a "microscopic bed of nails". DNA is either precipitated onto, or chemically bound to the arrays and the chip is pressed to the target tissue. Alternately, cells or clusters of cells can be forced onto the DNA-coated nanofiber array by centrifugation (the arrays are immobilized on the bottom of the centrifuge tube; McKnight et al. 2003). This approach has been more successfully employed for the introduction of DNA into animal cells as the plant cell wall barrier presents an additional hurdle for this approach. Nanofiber arrays represent a very young and inefficient technology for DNA introduction into plant cells but the approach seems logical and preliminary results look reasonable (Finer and Dhillon 2008).

1.5.5 Pollen Tube Pathway

In all fields of the sciences, premature claims are made which are often inadequately substantiated. The plant transformation sciences is certainly not exempt from this type of activity as new or more efficient methods for transformation are valuable and any success can accelerate career development. This "rush to publish" mentality has yielded numerous reports of new and exciting transformation methods which have not stood the test of time. The mixing of pollen with DNA and injecting DNA into the meristem and ovules have yielded some very provocative results which have not been repeated.

One method of transformation which enjoyed some major attention during the early days of plant transformation, and has seen resurgence, is transformation via the pollen tube pathway (Luo and Wu 1988). Soon after this early report with rice, the method was informally confirmed by others working with different crops. These follow-up early reports were never published. Over the years, transformation via the pollen tube pathway has been both ridiculed and praised but it has neither seen wide adoption nor been swept under the scientific carpet. This method is currently being actively used by one laboratory in China, which is quite aggressive with publication efforts (Yang 2009a, b).

For transformation via the pollen tube pathway, pollen is placed on the stigma and allowed to germinate and grow down the style to the ovary. The growing pollen tube contains the pollen nuclei and once the pollen tube grows down to the egg, one pollen nucleus fuses with the egg to form the zygote. When the pollen tube reaches the egg, the style is severed using a scalpel, supposedly leaving an open pollen tube. The success of this procedure is grounded in the concept of using a hollow pollen tube as a transport vehicle for direct DNA introduction into the freshly-fertilized egg. It is not clear whether the pollen tube is actually hollow. It is also unclear whether any DNA is able to enter the ovule. The timing of the cutting of the pollen tube and subsequent DNA introduction must be very precise, to have the DNA enter the cell with the pollen nucleus. The reported efficiency of the process is inexplicably high, considering that $<1\%$ of cells that contain DNA introduced via particle bombardment are able to integrate that DNA into their genome. Extensive analysis of soybean plants obtained through the pollen tube pathway suggests that this method is not reproducible (Shou et al. 2002).

In spite of these problems, the pollen tube pathway continues to receive positive validation in peer-reviewed literature, often in respected journals. Two recent reports (Yang 2009a, b) deserve special attention here, as the results should raise major concerns of scientific rigor. One of these papers reports a comparison of transformation via the "ovary drip" method (variant of the pollen tube pathway) and the pollen tube pathway (Yang et al. 2009a) while the other paper reports the results of a pollen tube pathway study (Yang et al. 2009b). In spite of reportedly using different methods and different maize lines in the two papers, the authors show the exact same image of a GFP-expressing root in both papers, as one piece of evidence for transformation. Further scrutiny of these papers reveals additional problems but

this one duplicated image is indicative of a basic problem with scientific accuracy. It appears that the pollen tube pathway method for DNA introduction has not yet been convincingly validated.

1.6 Evidence for Transformation

The premise behind DNA introduction into plant cells is the recovery of a phenotype from the activity of foreign gene(s). This phenotype is usually the ultimate goal of transformation scientists but some phenotypes can be difficult to discern; and additional means of confirming gene presence and function are necessary to determine whether a gene has been successfully introduced and is active. Proof of DNA presence and function should be relatively straightforward but the evidence can be misinterpreted. Tissues should be analyzed at a number of different levels: from the presence of the DNA, to the activity of the gene, to an altered phenotype in the transgenic plant or tissue.

1.6.1 DNA Presence

The simplest method of confirming the presence of foreign DNA is through the polymerase chain reaction (PCR). PCR is a very powerful and useful tool for amplifying fragments of DNA, using DNA primers designed to precisely bind to sites within a strand of DNA. The DNA used as a starting point for PCR can be used at low concentrations; and DNA quality issues are not paramount. PCR products are run on a gel or directly sequenced to show the appropriate size or composition of the products. PCR is an extremely reliable *preliminary* evaluation tool for determining the presence of DNA. However, because PCR allows the detection of extremely low concentrations of DNA, contamination is problematic and must be avoided or minimized. PCR alone is insufficient to prove transformation but it can be used as an efficient screening tool to select lines of interest for additional studies.

The best method for confirming the presence of introduced DNA is Southern hybridization analysis. For Southern analysis, DNAs are extracted from plant materials, digested, electrophoresed in a gel, blotted onto a membrane and finally hybridized to a labeled fragment of DNA which is complementary to the introduced DNA. The use of appropriate restriction enzymes generates precise hybridization results or patterns, which is strong evidence for successful DNA introduction. While PCR results suggest the presence of the introduced DNA, Southern analysis can also show DNA integration, which is a necessary indicator for successful transformation. In addition, Southern hybridization provides information on the number of copies of introduced DNA per nuclear genome, as well as the number of sites in the genome that the DNA integrates. To obtain meaningful results, Southern

hybridization analysis should be performed after careful assessment of appropriate restriction enzymes, which usually recognize one site within the introduced DNA. Use of the proper restriction enzyme yields sizes and patterns of hybridization signals that are unique for each transformation event. Digestion with restriction enzymes which are expected to generate uniformly sized fragments are of very limited value. The intensity of hybridization signal(s), as well as their size and shape, provides additional information on the validity of results. Although Southern analysis of PCR products is occasionally presented in the literature, results from this approach are prone to misinterpretation and should not be considered evidence for transformation.

1.6.2 Gene Expression

After the presence of the introduced DNA is verified, it is necessary to evaluate the expression of the introduced gene. This can be performed by testing for the presence of RNA, protein or an altered phenotype. Although it is not absolutely necessary to test for gene expression at all levels, an appropriate combination of analyses is usually needed. For the analysis of RNA, Northern hybridization or RT-RCR is used. For Northern analyses, the basic principles are the same as with Southern analysis but RNA is extracted instead of DNA. The hybridization signal should be a predicted size, which corresponds to the RNA transcript. For RT-PCR, DNA is generated from isolated RNA and the resulting amplicons are evaluated following electrophoresis. Again, the size and intensity of the resulting bands are important.

When the introduced DNA is a scorable marker gene, validation of gene expression is sometimes extremely simple and effective, especially if GFP is the marker. With the appropriate detection instrumentation, GFP can be directly observed in transgenic plant material. The fluorescent green color is usually unmistakable but inappropriate in-line filters and illumination can make interpretation difficult. Expression of GFP, as well as other transgenes, is dependant on the promoter used to regulate the gene. Transgene expression always shows some type of patterning, based on tissue type and inducibility. Even the use of "constitutive" promoters like the CaMV35S promoter show some patterns of gene expression. For example, expression occurs along developing cell lines, showing more intense activity in regions parallel to the longitudinal axis of the root, or close to the veinal tissue of the leaves. If the marker gene appears to weakly express throughout the tissue with no pattern, this suggests background expression or problems with the detection system.

If the gene of interest (GOI) is expected to give rise to a new phenotype, this analysis is ultimately needed. Some phenotypes can be difficult to gauge and any changes should also be clearly associated with the presence and expression of the transgene in primary transgenics and segregating progeny.

In the primary transgenics, all plants should contain the introduced DNA in a heterozygous state. Multiple copies of the transgene are commonly obtained but these copies often integrate into the same integration site, behaving like a single gene in the heterozygous condition. In the T1 progeny from single insertion events, the transgene segregates in a 1:2:1 ratio, with 25% of the progeny not containing the transgene, 50% heterozygous and 25% homozygous transgenic. When analyzing simply for the presence of the transgene, the transgene should be present in 75% of the T1 generation progeny (1:3). If ratios other than this are obtained, this suggests integration of the transgene into multiple sites (more than 75% of progeny contain the transgene) or lethality of the transgene in germ line cells or in the homozygous condition (less than 75% of progeny contain the transgene). If a very low percentage of the progeny contains the transgene, this suggests additional problems, which are a cause for concern.

1.7 Conclusions

The production of transgenic plants via transformation has tremendously impacted agriculture and the face of our planet. Through improvements in technology for DNA introduction, production of transgenics is no longer as limiting as it once was. Determination of potential GOIs as well as evaluation of transgenics is becoming a new bottleneck in plant biotechnology. However, improvements in DNA introduction methodology as well as developing predictable transgene expression models are still needed. Transformation efforts should not be curtailed until all plants of interest can be transformed with the same ease as *Arabidopsis*.

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