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Factors affecting somatic embryogenesis and transformation in modern plant breeding

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Abstract

Somatic embryogenesis and transformation systems are indispensable modern plant breeding components since they provide an alternative platform to develop control strategies against the plethora of pests and diseases affecting many agronomic crops. This review discusses some of the factors affecting somatic embryogenesis and transformation, highlights the advantages and limitations of these systems and explores these systems as breeding tools for the development of crops with improved agronomic traits. The regeneration of non-chimeric transgenic crops through somatic embryogenesis with introduced disease and pest-resistant genes for instance, would be of significant benefit to growers worldwide.

Keywords: *Somatic embryogenesis, transformation, plant growth regulators, Agrobacterium, microprojectiles*

1. Introduction

In plants, embryo-like structures can be generated from cells other than gametes (i.e. somatic cells) by circumventing the normal fertilization process, hence the term somatic embryos (Parrott, 2000). As somatic embryos are formed without any fertilization event they are genetically identical to the parent tissue and are therefore clones.

Somatic embryogenesis may be direct or indirect. Indirect somatic embryogenesis involves dedifferentiation of organized tissue into callus prior to embryo production whereas direct somatic embryogenesis involves production of embryo from organized tissue without an intervening callus phase (Slater *et al.*, 2003).

Irrespective of the mode of production, it has been argued that the anatomical and physiological features of somatic embryos are highly comparable to zygotic embryos (Cheng and Raghavan, 1985; Boxus, 1989; Gray, 1992; Zimmerman, 1993; Bandyopadhyay and Hamill, 2000). This claim is supported by the work of Bandyopadhyay and Hamill (2000) on *Eucalyptus nitens*, which revealed that both somatic and zygotic embryos have strong similarities in terms of their overall size, morphology and internal cellular organization. Zimmerman (1993) further argued that the morphological and temporal development of somatic embryos are very similar to that of zygotic embryos and that they both proceed through a series of distinct stages, namely globular, heart, torpedo and cotyledon or plantlet stages for dicotyledons (Zimmerman, 1993; Mandal and Gupta, 2002) and globular, elongated, scutellar and coleoptilar stages for monocotyledons (Gupta and Conger, 1999; Godbole *et al.*, 2002). These stages typically span a period of several days.

In dicotyledonous embryogenesis, small globular embryos initially form which then undergo isodiametric growth and establish bilateral symmetry. These then develop into the heart stage embryo in which both cotyledons and root and shoot meristems are clearly established. The development proceeds with the formation of torpedo and subsequently plantlet stages. The plantlets contain green cotyledons, elongated hypocotyls and developed radicals with very fine root hairs (Zimmerman, 1993).

In monocotyledonous embryogenesis, especially in graminaceous species, the transition from globular stage follows a series of events all occurring simultaneously. This includes the development of scutellum, initiation of the coleoptilar notch, and tissue differentiation with the development of embryogenic vascular system and accumulation of intracellular storage substances. At the final stages of maturation, the coleoptile undergoes enlargement and the embryo axis becomes more developed. The embryo axis develops laterally and parallel to the scutellum, while the root apical meristem is embedded and the shoot apical meristem develops externally and is protected by the coleoptile (Gray, 1996). The first leaf primordium appears at the base of the shoot apex while development of leaf primordia is preceded by the formation of root(s) from the root meristem. As the plantlet develops, numerous root hairs develop on the main root (Meinke, 1991).

1.1 How is Somatic Embryogenesis Used?

Somatic embryogenesis is a valuable tool in plant biotechnology and can be utilized in a number of ways (Zimmerman, 1993; Bandyopadhyay and Hamill, 2000; Saiprasad, 2001):

- For large-scale clonal propagation of elite cultivars it provides an alternative approach to conventional micropropagation.
- Synthetic (artificial) seed can be developed from somatic embryos potentially facilitating broad-acre direct seeding of elite cultivars or providing a means of moving germplasm in a less fragile form than *in vitro* plantlets.
- Embryogenesis via callus or secondary embryogenesis may assist in the application of gene transfer techniques for further genetic improvements.
- Somatic embryogenesis systems offer potential models for studying molecular, regulatory and morphogenetic events in plant embryogenesis.

1.2 The Advantages of Somatic Embryogenesis for Mass Propagation

Large-scale production of plants through the multiplication of embryogenic cell lines is the most commercially attractive application of somatic embryogenesis (Jiménez, 2001), and is the most practical application of this technique to benefit agriculture. It has many advantages over conventional micropropagation in this respect:

- It permits the culture of large numbers of somatic embryos, with up to1.35 million somatic embryos capable of being regenerated per litre of medium.
- During regeneration, root and shoot formation is simultaneous thus eliminating the need for a root induction phase as with conventional micropropagation.
- The mode of culture permits easy scale-up and subculture with low labour inputs.
- Cultures can be manipulated such that embryo formation and germination can be synchronized maximizing plant output while minimizing labour inputs.
- As with zygotic embryos, somatic embryos dormancy can be induced, hence long-term storage is possible.

1.3 Limitations of Somatic Embryogenesis

Even though somatic embryogenesis offers great potential, it also has some limitations. Firstly, the development of somatic embryos tends to be non-synchronous (Zimmerman, 1993; Zegzouti *et al.*, 2001), thus embryos of all stages can be present in one culture system. However, Fujimura and Komamine (1979) demonstrated that the development of carrot somatic embryos could be synchronized by grouping cell aggregates of similar size and density from suspension cultures using sieving and density gradient centrifugation. Although synchronization of somatic embryos can be achieved using these strategies, it appears that the percentage of somatic embryos regenerated is affected by the size of cell aggregates. Chee and Cantliff (1989) pointed out that a decrease in the size of cell aggregates led to a reduction in the percentage of somatic embryo formation in sweet potato. In spite of this phenomenon, selection of only the highly regenerable portion of a cell culture would still be a more efficient propagation system than conventional micropropagation. For example, the rate of somatic embryo formation in banana suspension cultures can be over 100,000 per mL of cells (Cote *et al.*, 1996). Thus, selection of a subgroup of cells and discarding the remainder would still provide many plants.

The second limitation is the stability of cell lines. Over a period of time, the proportion of cells that enter or complete embryogenesis decreases so that, eventually, regeneration may become impossible. This in fact could be an advantage as prolonged time in culture can lead to the accumulation of mutations (somaclonal variations), which can cause morphological abnormalities such as pluricotyledony, multiplex apex formation and fused cotyledons (Evans *et al.*, 1983). Thus, being forced to initiate new cultures as old ones lose regenerability may reduce the frequency of somaclonal variations. The two may in fact be linked with increasing mutations associated with an inability to regenerate. Working with suspension cultures of carrot, Evans *et al.* (1983) showed that frequently initiating new cultures and maintaining the cultures for less than one year resulted in the regeneration of phenotypically normal somatic embryos and plants. Apparently, somaclonal variation also occurs with conventional micropropagation hence new cultures are also initiated on a regular basis. It should be noted, however, that somaclonal variation could have tremendous potential for producing novel and useful varieties.

1.4 Role of Plant Growth Regulators (Pgrs) in the Development of Somatic Embryos

The most commonly used protocol for induction of embryogenesis involves the induction of callus in an auxin-supplemented medium and somatic embryogenesis upon transfer of callus to a medium low in growth regulators (Cheng and Raghavan, 1985; Smith and Krikorian, 1989; Smith and Krikorian, 1990; Gray, 1992; Zimmerman, 1993). The establishment and maintenance of embryogenic cultures of nearly all species has relied primarily on the manipulation of growth regulators (Smith and Krikorian, 1990). In particular, the presence of auxin promotes callus proliferation and inhibits differentiation while the removal or decrease in auxin allows somatic embryo development to progress. Morphogenetic changes can be observed upon transferring callus to an auxin-free medium (Cheng and Raghavan, 1985; Zimmerman, 1993). It appears that the removal of auxin from the medium provides the signal for the callus cells to embark on an organized pattern of growth. The fact that embryogenesis can occur upon withdrawal of growth regulators suggests that in the presence of auxin, the proembryonic masses (PEMs) within the culture system may already be "primed" to complete the globular stage of embryogenesis and that the PEMs may also contain products inhibitory to the progress of the embryogenesis program (Zimmerman, 1993). Consequently, the removal of auxin may result in the inactivation of genes responsible for the presence of these inhibitory products, enabling the embryogenesis program to proceed. The observation that some carrot cell lines were only able to develop to the globular stage in the continued presence of auxin also suggests that new gene products are needed for the transition to the heart stage and these new products are synthesized only when exogenous auxin is removed from the culture medium (Zimmerman, 1993).

Auxins are also known to be the principal agents responsible for the establishment of cell polarity (apical-basal axis). It has been suggested that the polar transport of auxin in early globular embryos is essential for the establishment of bilateral symmetry during plant embryogenesis (Liu *et al.*, 1993). For the induction of the process leading to polarity, relatively high levels of endogenous, free indole-3-acetic acid (IAA) may be necessary. However, once induction has occurred, those high levels of IAA must be reduced to allow the establishment of the auxin gradient. If the levels are too low or high or do not diminish after the induction, the gradient cannot be formed and thus somatic embryogenesis cannot progress (Jiménez, 2001). Failure in the establishment of proper gradient using inhibitors of cell

IAA efflux carrier proteins such as naphthylphthalamic acid (NPA) caused the formation of abnormal embryos in Indian mustard (*Brassica juncea*) (Liu *et al.*, 1993) and *Fucus distichus* (Basu *et al.*, 2002). Further, embryogenic carrot cells grown in the presence of 2,4-D contained high levels of IAA (Ribnicky *et al.*, 1996). High levels of endogenous IAA were also found in embryogenic callus cultures of maize and carrot and a loss in embryogenic competence of the calli due to prolonged time of culture occurred concomitantly with a reduction in the IAA levels. This suggests that auxin plays a role in the formation of apical-basal pattern in embryo development by influencing endogenous IAA (Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b), and that a proper distribution of auxin (a gradient) is required for the establishment of polarity, which is a critical event in plant embryogenesis (Sun *et al.*, 2004). Recently it has been proposed that auxin (2,4-D) may initiate somatic embryogenesis by inducing a stress response in plant cells (Pasternak *et al.*, 2002; Shinoyama *et al.*, 2004). Expression of stress related genes has been found in the early stages of embryogenesis, thus it has been proposed that this is an extreme stress response in cultured plant cells (Pasternak *et al.*, 2002). Prolonged incubation of the explants without subculture may also place the explants under stress since, nutrition depletion, accumulation of toxics compounds and water stress would occur after such a long incubation time. Therefore, it can be argued that stress *in vitro* cannot be entirely by the growth regulators but also the biotic and abiotic factors present in the culture medium.

Although auxins are known to be the principal agents responsible for cell polarity, other stimuli have an effect and hence influence the efficiency of somatic embryogenesis. For example, in white clover (*Trifolium repens* L.), cytokinin promoted the formation of embryogenic cells from the epidermis of immature zygotic embryos (Dodeman *et al.*, 1997), thus it was proposed that exogenous growth regulators modify cell polarity by interfering with pH gradients or electro potential at a cellular level. Consequently, it could be argued that the combined effect of multiple growth regulators and other components of the medium might influence both the establishment of cell polarity and the subsequent cellular processes leading to the formation and development of normal somatic embryos. That somatic embryogenesis commonly occurs using exogenous applications of auxin and its withdrawal, does not mean that this hormone alone is responsible as the plant tissue itself may synthesize endogenous auxins and other hormones.

Cytokinins are known to stimulate cells and, as such, they are also suitable candidates for induction of somatic embryogenesis and caulogenesis. For example, in some cases thidiazuron (TDZ) has stimulated *in vitro* shoot regeneration and somatic embryogenesis (Thinh, 1997; Mithila *et al.*, 2003; Srangsam and Kanchanapoom, 2003; Lin *et al.*, 2004). Like many synthetic plant growth regulators, TDZ was originally developed as a herbicide, in this case a cotton defoliant, with cytokinin-like qualities (Panaia *et al.*, 2004). It has been suggested that TDZ is more effective than other cytokinins used for somatic embryogenesis (Thinh, 1997; Lin *et al.*, 2004). The effects of TDZ occur at lower concentrations than other cytokinins and it has been suggested that it either directly promotes growth due to its own biological activity or through inducing the synthesis and/or accumulation of endogenous cytokinins or auxins. The latter could explain the effectiveness of TDZ as it may be mediating levels of endogenous auxin/cytokinin levels within the cultured tissue (Visser *et al.*, 1992; Panaia *et al.*, 2004).

The above details support the argument of Vasil and Vasil (1981) that "the initiation of shoots and embryos cannot be ascribed to any one plant growth regulator", although the most successful procedure appears to be the transfer of tissues from a medium containing auxin (commonly 2,4-D) to a medium devoid of this synthetic auxin or containing a very low concentration. It also appears that different species have different levels of sensitivity towards various plant growth regulators; hence, their response to embryogenesis is variable. For instance, in experiments with lucerne (*Medicago sativa L.*), 2,4-D either separately or in combination with kinetin or naphthalene acetic acid (NAA) and benzylaminopurine (BAP) could not stimulate the formation of embryogenic callus. However, this callus could be induced in medium containing IAA and zeatin (Kim *et al.*, 2004). In addition, as shown by Panaia *et al.* (2004) different responses can be obtained from species belonging to the same family. In the family Restionaceae, for example, *Desmocladus flexuosus* responded to BAP and TDZ while *Baloskion tetraphyllum* responded only to 2,4-D. This clearly indicates that the different requirements for plant growth regulators operate at species, and even at cultivar level. Hence, in any research on somatic embryogenesis, a range of plant growth regulators should initially be used, so that the optimal "stimulant" combination can be identified.

1.5 Explant, Plant Genotype and Culture Conditions are Crucial for Somatic Embryogenesis

Variations in *in vitro* response have been known to occur due to a number of different factors, such as basal medium (Zegzouti *et al.*, 2001), explant source (Sharma and Rajam, 1995; Haliloglu, 2002) and genotype (Radhakrishnan *et al.*, 2001; Kim *et al.*, 2003).

Various explants have been utilized to initiate somatic embryogenesis including anthers, pollen, ovaries, (Cheng and Raghavan, 1985; Songstad and Conger, 1986; Jayasree *et al.*, 1999), leaves (Cheng and Raghavan, 1985; Birhman *et al.*, 1994), petioles and stems (Cheng and Raghavan, 1985; Reynolds, 1986), immature and mature embryos (Smith and Krikorian, 1989; Gray, 1992), mature cotyledons (Venkatachalam *et al.*, 1999; Barry-Etienne *et al.*, 2002) and corms (Deo *et al.*, 2009).

Even though a variety of explants can be utilized, the correct developmental stage of the explants is also crucial for the initiation of embryogenic callus. Lu and Vasil (1982) demonstrated that when the explant stage in *Panicum maximum* was incorrect, only a soft, friable and translucent callus with no embryogenic potential was produced. In addition, young or juvenile explants produced more somatic embryos than older explants (Woodward and Puonti, 2001; Panaia *et al.*, 2004). As a further complexity, different explants tissues from the same mother plant produced embryogenic callus at different frequencies (Zhang *et al.*, 2001) and required different concentration of growth regulators for the induction of somatic embryos

(Sharma and Rajam, 1995). The different endogenous phytohormone levels of various explants tissues might be a factor influencing the requirements of exogenous growth regulators. As a general rule, the type and age of explants has an impact on somatic embryogenesis, and highlights the observation that young, dividing and possibly less differentiated cells are more likely to be stimulated towards the embryogenic pathway than older cells.

The effect of genotype on somatic embryogenic competence has been clearly shown. For example, out of the five cultivars of Hybrid Tea roses (*Rosa hybrida* L) investigated, somatic embryogenesis could only be induced in two (Kim *et al.*, 2003). A similar phenomenon has been observed in red clover (McLean and Nowak, 1998), peanut (*Arachis hypogea*) (Radhakrishnan *et al.*, 2001) and Chinese cotton (Zhang *et al.*, 2001). The presence of varying levels of endogenous phytohormones, particularly cytokinins, in different genotypes might influence their response to somatic embryogenesis. Wenck *et al*. (1988) observed that genotypes of orchard grass in which embryogenesis was difficult to induce contained considerably higher levels of endogenous cytokinins than embryogenic genotypes.

The recalcitrance of some species can be overcome by manipulating other media components (Birhman *et al.*, 1994). This has been substantiated by experiments of Panaia *et al*. (2004) whereby somatic embryogenesis in *Baloskion tetraphyllum* was achieved using half-strength MS salts with 0.22 mg/L 2,4-D, with approximately 14,000 somatic embryos obtained from 1 g of plant material. In another study, Samson *et al*. (2006) demonstrated that a two or four-fold dilution of the MS salts increased the development rate of *Coffea* embryogenic callus by 2.6 and 5.7, respectively, in comparison to full-strength MS salts. Conversely, Groll *et al*. (2002) showed that media with altered macro and micro nutrient salt concentrations affected the development and germination capability of cassava somatic embryos, with half-strength and full-strength MS medium proving superior for development and germination compared to quarter-strength MS. This clearly demonstrates that medium modifications, in particular manipulating the concentrations of inorganic salts and vitamins, can have a significant effect on somatic embryogenesis possibly through altering the osmotic potential of the medium.

In addition to modifying medium components, Gairi and Rashid (2004) showed that by subjecting explants or callus culture to auxin (2,4-D) treatment for several days followed by their transfer to medium containing TDZ, a previously non-responsive or recalcitrant cultivar of rice could be induced to become responsive to somatic embryogenesis. This phenomenon has also been reported in taro (Deo *et al.*, 2009). This introduces another variable in the development of methods to induce somatic embryogenesis, that is, brief “pulses” on one medium followed by transfer to another.

The success in initiating embryogenic callus, somatic embryos and the subsequent recovery of viable plants is not readily achievable for many species. Induction may, in fact, demand long and complex treatments or procedures where non-embryogenic cells can be induced to an embryogenic state by a variety of procedures including treatment with plant growth regulators and various other chemicals and manipulations to light, temperature and pH. Therefore, in order to determine the effective conditions for somatic embryogenesis in different species, the required conditions must be determined empirically by manipulating the many factors that contribute to the culture conditions (Jiménez, 2001).

1.6 The Effect of Light and Activated Charcoal

Many plants are rich in phenolic compounds. Therefore, after tissue injury, such compounds will be oxidized by polyphenol oxidases and the tissue will become brown. The oxidation products are known to not only darken the tissue, but also to inhibit activity of various proteins which may have an inhibitory effect on somatic embryogenesis (Evans *et al.*, 1983).

Smith and Krikorian (1990) reported that somatic embryogenesis in carrot failed to occur under continuous light unless activated charcoal filter papers were used. Growth under white light had been associated with elevated phenolic production and an increased level of abscisic acid (Evans *et al.*, 1983; Smith and Krikorian, 1990). Activated charcoal removes inhibitors of embryogenesis, in particular phenylacetic acid, benzoic acid derivatives and other colourless toxic compounds by adsorption (Drew, 1972; Srangsam and Kanchanapoom, 2003). Moreover, activated charcoal also has been shown to absorb 5-hydroxymethylfurfural, an inhibitor formed by the degradation of sucrose during autoclaving, as well as substantial amount of auxins and cytokinins. Consequently, apart from removing inhibitors that would prevent growth, it may also adsorb and reduce the levels of growth regulators that would otherwise stimulate callus initiation, growth and proliferation. Therefore, it has been suggested that cultures should be maintained in reduced light intensity or in darkness, as this will minimize the production of inhibitory compounds from tissues in the culture medium (Evans *et al.*, 1983). In addition, this will also minimize or negate the inclusion of activated charcoal in the medium, thus ensuring that the potential of the growth regulators present in the medium will not be compromised.

1.7 The Effect of Other Biochemical Factors on Somatic Embryogenesis

Certain bioactive compounds such as the amino acids, glutamine, proline and tryptophan and polyamines such as putrescine have been identified as enhancers of somatic embryogenesis in some species. Their efficacy in embryogenesis has been attributed to their contribution to various cellular processes such as improving cell signaling processes in various signal transduction pathway (Lakshmanan and Taji, 2000), as precursor molecules for certain growth regulators (Siriwardana and Nabors, 1983; Ribnicky *et al.*, 1996; Jiménez and Bangerth, 2001a; Jiménez and Bangerth, 2001b) or regulators of DNA synthesis (Kevers *et al.*, 2000; Astarita *et al.*, 2003). Some species does not require these additives.

Some researchers have emphasized that the inclusion of complex organic extracts, such as coconut water (CW), taro extract (TE), potato extract (PE), corn extract (CE) and papaya extract (PAE) are essential for somatic embryogenesis in some species (Ichihashi and Islam, 1999;

Islam *et al.*, 2003; Rahman *et al.*, 2004). Moreover, it has also been proposed that these organic extracts are either non-mutagenic or less mutagenic in comparison to conventional growth regulators and, as such, their incorporation in the culture media may minimize somaclonal variations (Lam *et al.*, 1991). However, it can also be argued that organic extracts are undefined components and as such it is not possible to: (i) determine which particular constituent of the extract promotes somatic embryogenesis, and (ii) ensure consistency in the actual extract each time it is prepared.

1.8 Somatic Embryo Maturation

An embryogenesis system requires the following steps, which occur in succession: initiation of embryogenic callus from vegetative tissues or cells, maintenance and multiplication of embryogenic cell lines, somatic embryo formation and maturation and finally conversion (germination) of somatic embryos into viable plantlets (Zegzouti *et al.*, 2001).

Maturation is regarded as an essential stage of embryogenesis since the frequency of plant recovery is high from mature embryos. Embryo maturation is a culmination of the accumulation of carbohydrates, lipids and protein reserves, embryo dehydration and a reduction in cellular respiration (Trigiano and Gray, 1996). Thus, maturation is a preparatory stage for embryos for effective germination as Etienne *et al*. (1993) stated, "maturation is a transitory, frequently indispensable stage between embryo development and embryo germination phases"; consequently, bypassing the maturation phase will result in precocious germination of embryos causing a significant reduction in viable plantlets.

Dehydration was hypothesized to be critical for maturation (Etienne *et al.*, 1993). As such, restricting water uptake using osmoticum was studied (Etienne *et al.*, 1993; Attree *et al.*, 1995; Gutmann *et al.*, 1996) for its ability to support development of plant embryos while at the same time suppressing precocious germination. Permeating osmoticum, such as sucrose, is frequently used to reduce the water potential of the culture medium resulting in water stress thereby promoting embryo development during *in vitro* culture. However, Attree *et al*. (1995) argued that during prolonged culture, such osmoticum would be taken up by the plant cells leading to osmotic recovery. In contrast, non-permeating osmoticum, such as polyethylene glycol-4000 (PEG-4000), can continue to restrict water uptake and so provide a longer-term drought stress during embryo development. In addition, the rate of desiccation also has an impact on the germination and conversion of somatic embryos into plantlets. For example, rapid desiccation of immature somatic embryos of *Hevea* improved their germination capacity, but their continued development into plantlets was low. In contrast, slow desiccation led to enhancement of germination and was more effective in stimulating conversion into plantlets (Etienne *et al.*, 1993). Slow desiccation resulted in substantial accumulation of starch and protein reserves required for continued development of immature embryos in comparison with rapid dehydration. Therefore, desiccation could be used to enhance germination when the embryo approaches physiological maturity (Etienne *et al.*, 1993). Moreover, Attree *et al*. (1995) emphasized that the ability to dry somatic embryos reduces large-scale production costs by providing a means of storing somatic embryos that are produced continuously throughout the year. They can then be germinated synchronously to provide plants of uniform age and size for planting later during a suitable growing season.

Gutmann *et al*. (1996) observed that exogenously supplied abscisic acid (ABA) was an important component of maturation medium for *Hybrid larch* somatic embryos. In the absence of ABA, maturation resulted in poorly developed somatic embryos often exhibiting abnormal morphology, non-synchronous development and precocious germination. Subsequently, these somatic embryos had the lowest capacity for germination and plantlet development. In contrast, the presence of ABA in the maturation medium promoted the development of higher quality somatic embryos in large quantities. Under appropriate conditions these somatic embryos germinated and developed into plantlets at a high frequency. It can therefore be concluded that for a somatic embryogenesis system to be practically applied, high frequency embryo formation is of little value unless a large proportion of these embryos are capable of developing into normal plants (Venkatachalam *et al.*, 1999).

1.9 Genetic Transformation in Plants

Transformation is the introduction of exogenous DNA into plant cells, tissues or organs employing direct or indirect means (Alves *et al.*, 1999). Indirect gene transfer involves the introduction of exogenous DNA by a biological vector such as *Agrobacterium*, whereas direct gene transfer involves introduction of exogenous DNA by physical or chemical processes such as electroporation, polyethylene glycol mediated DNA uptake, microinjection, silicon carbide fibres and microprojectile bombardment (Taylor and Fauquet, 2002). Virtually any desirable trait found in nature can, in principle, be transferred to any plant species by transformation (i.e genetic modification) hence the term transgenesis (Betsch, 1994). Further, with DNA synthesis technology becoming more sophisticated, the transgene can be an entirely synthetic sequence.

1.10 Applications of Transformation

One of the major objectives of plant transformation has been to solve agricultural problems without environmental damage (Alves *et al.*, 1999). Following the advent of pioneer transgenic plants containing marker genes, todays transgenic crops are incorporated with commercial value genes and the most commonly used traits are herbicide tolerance (35 %), product quality (20 %), insect resistance (18 %), virus resistance (11 %), fungal resistance (3 %), nematode and bacterial resistance and marker or reporter genes (13 %) (Alves *et al.*, 1999; Vines, 2001; Koichi *et al.*, 2002). Other valuable genes are used to generate nutritionally enhanced crops with altered carbohydrate, starch, protein or lipid characteristics, higher vitamin or anti-oxidants content, improved taste, increased shelf-life and better ripening characteristics. Plants are also envisaged as "manufacturing facilities", hence considerable transformation work is being carried out to generate transgenic plants to produce large quantities of materials including therapeutic proteins and vaccines, textile fibres, oils for industrial use, detergents and

lubricants (Vines, 2001; Becker and Cowan, 2006), hence this category of transgenic plants are called "pharma crops" (Bauer, 2006). Another area of interests in transgenesis is biofuels. Plants are being modified to make it highly susceptible to cellulase digestion. The glucose monomers generated from cellulose could be used in large-scale ethanol production.

2. Methods of Transformation

Predominantly *Agrobacterium* and microprojectile particle bombardment (biolistics) are employed in plant transformation. The former has the advantages of low frequency of transgene rearrangement and low copy number of transgene integration (Sharma *et al.*, 2005), thus minimizing the incidence of gene silencing. In general, dicotyledonous species are generally more amenable to *Agrobacterium*-mediated transformation than monocots (Hagio, 1998). Consequently, biolistics is a logical starting point in the development of a transformation system for monocots. One of the disadvantages of particle bombardment is that it can result in high transgene copy number and a high frequency of transgene rearrangement, which may lead to transgene silencing or co-suppression (O'Kennedy *et al.*, 2001). With the advent of chemotatic chemicals such as acetosyringone, *Agrobacterium* transformation of monocots such as rice, wheat and banana has materialized. Regardless of the method employed, every stable transformation process demands the simultaneous occurrence of two independent biological events: the stable insertion of the transgene into the plant genome and regeneration from those cells in which this has occurred, producing a non-chimeric transgenic plant (Alves *et al.*, 1999).

2.1 Microprojectile Bombardment

The gene gun currently in use is the PDS-1000/He™ device, which is powered by a helium gas pressure breaking a rupture disc, which then accelerates a macrocarrier, upon which DNA-coated microcarriers have been dried. This system, allows better control over bombardment parameters, distributes microcarriers more uniformly over target cells, is gentler to target cells, is more consistent between bombardments and yields several folds more transformations (Hagio, 1998). Another simple and inexpensive particle bombardment device for delivery of DNA into plant cells is the Particle Inflow Gun (PIG) (Finer *et al.*, 1992), in which the DNA coated-microprojectiles are accelerated directly in a pressurised stream of helium rather than being supported by a macrocarrier.

2.2 Factors Affecting Biolistic Transformation

Stable transformation of plants using biolistics requires the penetration of cells by microprojectiles, integration of the transgene of interest into the host plant genome followed by subsequent expression, and finally, continued growth of the transformed cells and regeneration of plantlets (Russell *et al.*, 1992). Different plant species may behave differently throughout any of these steps. Several factors have been reported to affect the efficiency of particle and DNA delivery into the plant cells and subsequent transient expression and stable integration of the transgene. Microcarriers (or microprojectiles) may consist of different materials and be of different sizes. The two most commonly used microcarriers are gold and tungsten. In general, gold particles are preferred as they are more uniform in size and shape than tungsten resulting in less cell damage. Further, tungsten particles may undergo surface oxidation, which can alter DNA binding and catalytically degrade DNA bound to them (Sanford *et al.*, 1993) and cause toxicity problems in certain species (Russell *et al.*, 1992). However, tungsten should not be immediately excluded as an option for biolistics as it is considerably less expensive and perfectly satisfactory for some applications.

Small changes in the diameter of the particle can have an impact on particle momentum, the quantity of DNA the particle carries and the size of the lesion produced in plant cells, hence affecting cell survival (Klein *et al.*, 1988; Häggman and Aronen, 1998; Janna *et al.*, 2006). There is a range of sizes available from 0.6 µm up to 1.6 µm. It has been demonstrated that transient GUS expression is much greater using 1.0 µm rather than 1.6 µm particles (Tian and Seguin, 2004). Particles smaller than 1.0 µm are usually reserved for small cells such as microalgae, yeast and bacteria.

Multiple bombardments of the same target tissue have been trialed in an attempt to transform a greater number of cells. This strategy has been shown to both increase (Klein *et al.*, 1988) and decrease (Janna *et al.*, 2006) transient expression. The decrease was most likely due to excessive damage.

Other biolistic parameters shown to affect transformation efficiency include helium pressure, macrocarrier flight distance to baffle screen, distance from baffle screen to target tissues and vacuum pressure. The factors which affect the delivery and transient expression of the gene have been studied and optimized for different species (Quoirin *et al.*, 1997; Marchant *et al.*, 1998; Deroles *et al.*, 2002; Janna *et al.*, 2006). It is apparent that optimal parameters for transformation efficiency must be arrived at empirically for each species.

Use of osmoticums such as mannitol, sorbitol, sucrose and myo-inositol, have also been reported to enhance biolistic gene transfer and subsequent reporter gene expression in some species. For example, Clapham *et al.* (1995) reported a five to twelve fold increase in reporter gene expression in embryogenic cell cultures of *Picea abies*, which were treated with myo-inositol, before and after bombardment. Moreover, Ye *et al.* (1990) also reported a 20-fold increase in transformation efficiency in chloroplasts using sorbitol and mannitol in the bombardment and incubation medium. It has been proposed that osmotic treatment causes cells to become plasmolysed and by reducing turgor pressure, extensive damage to cell membrane is minimized and the leakage of the protoplasm is prevented when the microcarriers penetrate the cells (Hagio, 1998; Marchant *et al.*, 1998; Santos *et al.*, 2002). In addition, since plasmolysed cells are less rigid, particle penetration may also be improved (Hagio, 1998). Osmoticums are not required for all species and for those where it is used, the type and concentration is variable (Hagio, 1998).

2.3 *Agrobacterium*-Mediated Transformation

Agrobacterium tumefaciens is a gram-negative soil

bacterium, which causes crown gall disease in some plants species. They harbour large tumor inducing (Ti) plasmids of more than 200 kb in size, which contain the oncogenic, opine synthesis and virulence (*vir*) genes necessary for the establishment of infection and also opine catabolism genes allowing the bacterium to catabolise that particular set of opines (de la Riva *et al.*, 1998). In addition, Ti-plasmids also contain genes for conjugative transfer of plasmids between *Agrobacterium* (Opabode, 2006). Ti-plasmids are classified as octopine, nopaline or agropine based on the type of opine produced and excreted by the tumors they induce. Thus, an *Agrobacterium* strain with an octopine Ti-plasmid will induce tumors that synthesize octopine and also encode genes required to utilize octopine as a source of carbon and nitrogen (Knauf *et al.*, 1983).

Within the Ti plasmid is a region that is copied and transferred to the plant cell, called the transfer or T-DNA. It contains two types of genes: the oncogenic genes encode enzymes involved in the synthesis of auxins and cytokinins which are responsible for tumor formation and the genes encoding enzymes for the synthesis of opines. Opines are exclusively utilized by *Agrobacterium* as a carbon and nitrogen source (Tzfira and Citovsky, 2006). The T-DNA region is flanked at each end by 25 bp T-DNA borders, the left and the right border, which are essential for T-DNA transfer. They are the target of Vir D1/Vir D2 border specific endonucleases, which process T-DNA from Ti-plasmid (Gelvin, 2003) and function in a *cis*-acting fashion (de la Riva *et al.*, 1998).

The virulence genes (*vir* genes) located on the Ti-plasmid encodes a set of proteins responsible for excision, transfer and integration of T-DNA into the plant genome. The virulence region (30 kb) is organized in six operons: *vir* A, *vir* B, *vir* D and *vir* G are essential for the T-DNA transfer while *vir C* and *vir* E increase the efficiency of T-DNA transfer. The number of genes per operon differs: *vir* A, *vir* G and *vir* F have only one gene, *vir* E, *vir* C and *vir* H have two genes, *vir* D and *vir* B has four and eleven genes, respectively (de la Riva *et al.*, 1998).

2.4 Using Agrobacterium as a Tool for Plant Transformation

Most of the protocols established to date for plant transformation via *Agrobacterium* have relied on the innovation of binary vectors and virulence helper Ti-plasmids (Figure 1) (Tzfira and Citovsky, 2006). The binary vector strategy is based on the fact that the *vir* genes and T-region could be separated into two different replicons. When these replicons are within the same *Agrobacterium* cell, the products of *vir* genes could operate in *trans* on the T-region to effect T-DNA processing and transfer to a plant cell provided the DNA is placed between two correctly oriented T-DNA borders. Therefore, the native T-DNA of the Ti-plasmid can be removed to prevent tumor formation (disarmed) to produce a virulence helper Ti-plasmid. The T-DNA is located on another smaller plasmid, the binary vector that contains multiple cloning site, markers for selection and maintenance in both *E. coli* and *Agrobacterium*, plant selectable marker gene between the right and left borders of T-DNA and origin of replication (ori) that permits the maintenance of plasmid in *E. coli* and *Agrobacterium*. In the binary Ti vectors, the plant selectable marker genes are placed near the left border (LB) while the gene of interest is placed near the right border (RB). Since during T-DNA transfer the RB precedes the LB, therefore placing the gene of interest closer to the RB ensures that it will be transferred before the selectable marker gene. Consequently, creation of transgenic plants containing only the selectable marker gene could be avoided if the bacterium to plant T-DNA transfer is interrupted (Hellens *et al.*, 2000). The binary vector can be cloned in *E. coli* and transformed into an *Agrobacterium* strain containing a helper plasmid (Gelvin, 2003).

2.5 Factors Affecting *Agrobacterium*-Mediated Transformation

The transfer of T-DNA and its stable integration into the plant genome is influenced by several factors. Plant tissues require an optimum density of *Agrobacterium* cells for a high frequency of transformation. In wheat inflorescence tissue, a cell density of less than 1.0 OD (optical density) resulted in very low transient GUS expression whereas at densities of 1.0-1.5 OD very high GUS expression was achieved (Amoah *et al.*, 2001). A cell density above 1.5 OD significantly reduced the frequency of transformation. In banana cell suspensions, although high inoculum densities (1.0 and 2.0) resulted in high transient gene expression, cell death was also high (Khanna *et al.*, 2004) indicating that densities giving high transient expression may not translate into a high number of stable transformants. In contrast to the two previous examples, use of a 0.5 OD of *Agrobacterium* resulted in severe necrosis in cauliflower (*Brassica olerecea* var. *botrytis*) explants, but a 1:20 dilution of the same culture significantly increased transient GUS expression (Chakrabarty *et al.*, 2002). It has also been demonstrated that the effect of *Agrobacterium* cell density on transformation efficiency can be manipulated by varying the duration of co-culture time, with high cell density and reduced co-culture time increasing the frequency of transformation (Amoah *et al.*, 2001; Opabode, 2006) Therefore, it is essential to optimize the inoculum levels of *Agrobacterium* so that cell necrosis is minimized while a high level of T-DNA transfer is maintained.

The use of certain compounds such as acetosyringone and pluronic acid F68 has been reported to increase the efficiency of transformation. Acetosyringone (a low molecular weight phenolic compound) is used as an exogenous stimulant for the induction of *vir* genes (Chakrabarty *et al.*, 2002; Opabode, 2006). Some monocotyledonous plants produce only very low levels or none of these types of phenolic compounds and thus cannot activate the vir genes of *Agrobacterium* (Suzuki *et al.*, 2001). Therefore, exogenous application of acetosyringone in co-cultivation medium has been recommended to overcome this problem. Incorporation of acetosyringone in the bacterial culture medium (pre-induction) and in the medium in which *Agrobacterium* is co-cultured (co-cultivation) with plant cells increased T-DNA transfer into banana suspension cells (Khanna *et al.*, 2004) and in *Agapanthus praecox* ssp. *orientalis* (Suzuki *et al.*, 2001). There is a range of concentrations within which acetosyringone is effective; a minimum at which it is effective and a maximum at which it becomes bacteriostatic (Amoah *et al.*, 2001). Pluronic acid F68 is a

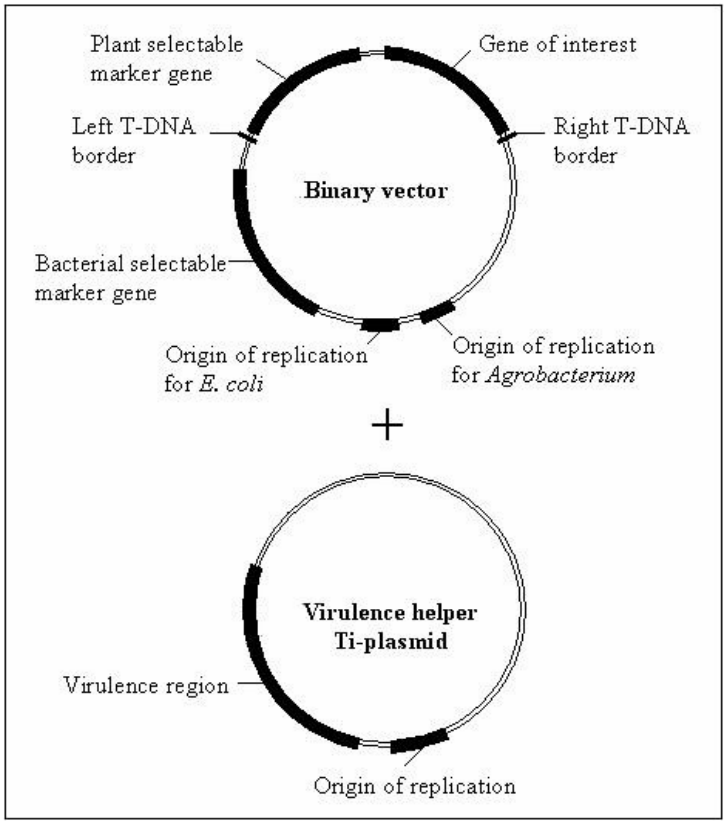

Figure 1. Diagram depicting the binary vector strategy used in plant transformation by *Agrobacterium tumefaciens.* Typical binary vector system consists of a small binary vector and a large virulence helper Ti-plasmid**.** The binary vector has a plant selectable marker gene and the transgene between the left and right borders. *E. coli* origin of replication allows initial cloning steps to be done in *E. coli. Agrobacterium* containing the virulence helper Ti-plasmid can be transformed with the binary vector and transformants selected using the bacterial marker gene on the binary vector. The virulence helper plasmid contains virulence genes necessary for T-DNA transfer to plant cells.

surfactant. It facilitates *Agrobacterium*–plant cell attachment and also reduces the effect of substances that inhibit *Agrobacterium* attachment, thus enhances T-DNA delivery by *Agrobacterium* (Opabode, 2006).

Even though successful transformations have been achieved by *Agrobacterium* in recalcitrant monocotyledonous crops such as maize, rice, wheat, sorghum and banana, the frequency of transformation may still be quite low. It has been reported that cells become necrotic after being infected by the bacterium (Carvalho *et al.*, 2004). Rinsing co-cultured cells with timentin and incorporating it in the selection medium has been shown to be effective in eliminating *Agrobacterium* from plant cells (Carvalho *et al.*, 2004). In addition, allowing explants to grow without selection except against *Agrobacterium* after co-cultivation could help them recover from infection and thus reduce cell necrosis (Carvalho *et al.*, 2004; Bhalla and Singh, 2008). Further, providing heat shock to plant cells before co-culturing with *Agrobacterium* has been shown to increase the viability of cells, resulting in the recovery of large number of transgenic plants (Khanna *et al.*, 2004). Heat shock causes plant cells to release heat shock proteins (Hsp) as a means to resist stress (Wang *et al.*, 2004). It would appear that activation of heat shock proteins prior to Agro infection enhances their immunity against *Agrobacterium.*

Modulation of the plant response such as inhibiting the programmed cell death (PCD) response has also shown to increase the frequency of stable transgenics (Khanna *et al.*, 2007). PCD is triggered by plants response to both biotic and abiotic stress. Upon pathogen attack, PCD is induced by transcribing apoptotic genes to kill infected cell, hence eliminating the spread of infection (Dickman *et al.*, 2001). Khanna *et al*. (2007) demonstrated that by expressing the animal antiapoptosis genes *Bcl-xL*, *Bcl-2* 3′ untranslated region, and *CED-9* in banana suspension cells, improved the frequency of viability and transformation by 90%.

A more difficult problem to overcome would be a deficiency in host genes required for T-DNA transfer and integration (Nam *et al.*, 1999; Zhu *et al.*, 2003). These groups reported that Arabidopsis mutants resistant to *Agrobacterium* transformation (*rat* mutants) failed to express genes such as chromatin structural and remodeling genes, and genes encoding proteins implicated in nuclear targeting, cell wall structure and metabolism, cytoskeleton structure and function, and signal transduction.

Agrobacterium strains differ in their ability to infect plants and transfer T-DNA (Suzuki *et al.*, 2001; Khanna *et al.*, 2004). For example, in sunflower (*Helianthus annuus* L.) genotypes, cv. Capella and SWSR2 inbred line, *Agrobacterium* strain, LBA4404 was more effective with the former cultivar while the strain GV3101 was effective with the latter cultivar (Mohamed *et al.*, 2004). In cauliflower (*Brassica oleracea* var. *botrytis*), a high level of GUS expression was observed in explants infected with *Agrobacterium* strain GV2260, while co-cultivation with

LBA4404 strain resulted in very low levels of expression (Chakrabarty *et al.*, 2002). Generally, the use of a highly virulent strain of *Agrobacterium* or using super-binary vectors enhances the frequency of transformation in recalcitrant crops (de la Riva *et al.*, 1998).

The type of explants (target material) is also important. *Agrobacterium* require cells that are actively dividing for gene transfer to occur (Okada *et al.*, 1986). Therefore, embryogenic callus and suspension cells are frequently used as a suitable target. Like any method for the production of transgenic plants the target material must also be suitable for regeneration so that transgenic plants can be recovered (de la Riva *et al.*, 1998).

2.6 Regulatory Sequences For Transgene Expression

Promoters are an essential element in transformation, as they are required to drive expression of both the selectable marker gene and the gene of interest. Numerous promoters are currently available, the most common of which are listed in Table 1.

Since high levels of expression are frequently desirable, constitutive promoters are commonly used. Constitutive promoters cause gene expression throughout the life of the plant in most tissues. The most widely used constitutive promoter is CaMV 35S derived from cauliflower mosaic virus (Alves *et al.*, 1999). Häggman and Aronen (1998) reported very high transient GUS expression in Scots pine embryogenic cultures using the constitutive CaMV 35S promoter. Similar results have also been reported by Zipf *et al*. (2001). Even though a high level of transient expression is not the sole determining factor for stable transformation, it is a useful indicator for the development of stable transformation for many species (Tian and Seguin, 2004).

However, Häggman and Aronen (1998) argued that the effects of promoters are dependent on both the tissue type and the species. Consequently, for successful expression of the gene of interest in the target tissue/plant, a thorough and careful selection of a suitable promoter is essential (Zipf *et al.*, 2001).

2.7 Marker and Reporter Genes are Essential in Transformation

When transformation protocols are developed, a selectable marker gene and a reporter gene are included in the transformation vector (Sharma *et al.*, 2005). After development of the protocol, the reporter gene is replaced with the gene of interest. Selectable markers consist of genes encoding enzymes capable of inactivating a toxic substance, commonly an antibiotic or herbicide. Alternatively, the toxic substance targets the active site of a protein vital for cellular process and the selectable marker gene codes for a variant of this vital protein, which is not affected by the toxin (Weeks *et al.*, 2000). Thus, selectable markers allow survival of the few cells in which the transgene has integrated facilitating efficient selection of transformed cells (Rao and Rohini, 2003; Sharma *et al.*, 2005). The most commonly used selectable marker genes are: *npt II* gene (neomycin phosphotransferase II), which confers resistance to kanamycin or G 418 (geneticin), *hph* gene (hygromycin phosphotransferase) that confers resistance to hygromycin B, and *bar* gene (phosphinothricin acetyltransferase) that confers resistance to the herbicide phosphinothricin (Rao and Rohini, 2003).

For each transformation and regeneration system, a minimum level of a selective agent, which can fully inhibit the growth of non-transformed cells, should be determined by use of a "kill curve" (Yang *et al.*, 1999). A concentration of selective agent greater than that necessary is likely to result in reduced transformation efficiency. While transgenic cells might be resistant to the selective agent, it can sometimes be the case that the selective agent or its metabolite negatively interferes with regeneration.

Positive selection has been proposed as a means of avoiding interference with regeneration by toxic agents and also removing the stigma of having antibiotic and herbicide resistance genes released into the environment (Yoo *et al.*, 2005). Positive selection relies on providing an essential nutrient in a form that can only be metabolized by the protein encoded by the marker gene; for example, the phosphomannose isomerase (PMI) system which uses mannose as the selective agent. Transgenic plants expressing the enzyme PMI encoded by the *man* A gene from *E. coli* are able to convert mannose-6-phosphate to fructose-6-phosphate which is metabolized through glycolysis (Joersbo, 2001; Reed *et al.*, 2001; Penna *et al.*, 2002).

Reporter genes are included in transformation vectors for two reasons: (i) to enable easy identification of potential transformants during the development of a transformation protocol and (ii) as a means of assessing tissue specificity and quantifying activity of promoters or other transgene systems. Reporter genes such as chloramphenicol acetyltransferase (CAT), β-glucuronidase (GUS), nopaline synthase and octopine synthase are bacterial in origin (Sharma *et al.*, 2005), while others have been derived from insects (luciferase, LUX) and jellyfish (green fluorescent protein, GFP). The most commonly utilized reporter gene is *uid* A, which encodes for the enzyme β-glucuronidase (GUS). In the presence of the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), GUS cleaves glucuronic acid producing a soluble colourless indoxyl group which is rapidly oxidized and dimerizes to produce an insoluble indigo precipitate. GUS is easily visualized and the enzyme is relatively stable. However, the assay is destructive and GUS-positive explants cannot be recovered (Alves *et al.*, 1999). The "green fluorescent protein" (GFP) is another useful reporter gene for plant and animal systems (Alves *et al.*, 1999). The gene encoding GFP was originally derived from a bioluminescent jellyfish, *Aequorea victoria*. The coding region was modified such that the protein is able to emit green fluorescence upon excitation under blue or UV light without any additional substrate. It is also a non-destructive assay (Miki and McHugh, 2004).

3. Combining Somatic Embryogenesis and Transformation for Crop Improvement

Efficient plant transformation systems require target tissue that is competent for proliferation and regeneration into plantlets. Direct organogenesis from the mature organs of monocotyledons and many dicotyledons occurs infrequently if at all. In most instances, a large population of totipotent cells in the form of callus or suspension cells is multiplied prior to transformation (Koichi *et al.*, 2002). Embryogenic cultures are the most commonly used target

Table 1. List of common promoters used in transformation systems.

Promoter	Derivation †	Specificity ‡	Reference
nos	*Agrobacterium* nopaline synthase	Developmentally regulated, organ specific	(An *et al.*, 1988)
CaMV 35S	*Cauliflower mosaic virus* encoding 35S RNA	Constitutive	(Benfey *et al.*, 1990)
Ubi-1	Maize polyubiquitin	Strongest in meristematic and vascular tissue, activity increased by heat shock	(Christensen *et al.*, 1992)
rbcS	Rubisco small subunit (from several monocot and dicot species)	Light induced expression in leaves	(Sugita *et al.*, 1987; Kyozuka *et al.*, 1993)
Act	Rice actin	Constitutive	(Zhang *et al.*, 1991)

† Derivation: the organism and protein whose corresponding gene the promoter was derived from.
‡ Specificity: tissue in which promoter is active and physiological requirements for activation if applicable.

tissue for high frequency recovery of non-chimeric transgenic plants (Taylor and Fauquet, 2002). Such cultures possess a high proportion of cytoplasmically rich, actively dividing cells, which provide high levels of transient expression and high frequency transgene integration (Mahn *et al.*, 1995). In addition, it is believed that these cells are better able to overcome the stress induced by transformation (Santos *et al.*, 2002).

Somatic embryogenesis and genetic transformation are extremely valuable tools in plant biotechnology. Efficient embryogenic systems facilitate the generation of large numbers of transgenic lines required for screening the desired trait. At present, it is generally necessary for commercial plant biotechnology companies to generate hundreds of transgenic lines, all containing the same transgene, in order to select one line which has the new desired trait while retaining the superior agronomic traits of the parent plant. Although such screening processes are tedious and inevitable, the probability of getting non-chimeric transgenics via somatic embryogenesis is exceptionally high.

Despite success in improving the efficiency of transient gene expression, the recovery of large number of stable transgenics in a relatively short time frame is still a major hurdle in transformation technology. It could be that cells being transformed take more time to recover from the trauma inflicted by the vector (transforming agent) or integration of transgenes into transcriptional active sites in host genome coding for essential metabolic enzymes are offsetting their regeneration potential. As a further complication, the selective agents used could impose additional stress on the transformed cells. Advancement in swift recovery of stable transgenics with no escapes would be phenomenal to molecular plant breeding.

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