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Factors Affecting *Agrobacterium*-mediated Transformation of Plants

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ABSTRACT

Plant transformation technology has become a versatile platform for cultivar improvement as well as for studying gene function in plants. The development of an efficient method for genetic transformation is a prerequisite for the application of molecular biology to the improvement of a given crop species. *Agrobacterium*-mediated genetic transformation is the dominant technology used for the production of genetically modified transgenic plants. Extensive research aimed at improving the molecular machinery of *Agrobacterium* responsible for the generation and transport of the bacterial DNA into the host cell has resulted in the establishment of many recombinant *Agrobacterium* strains and technologies currently used for the successful transformation of numerous plant species. Many factors influencing *Agrobacterium*-mediated transformation of plants have been investigated and elucidated. These factors include bacterial strains and cell density, plant species and genotype, plant growth regulators and antibiotics, explant, explant wounding, light and temperature. Before attempting stable transformation of any new species, it is useful to optimize the factors influencing transformation efficiency, as this can reduce future costs in labor and materials. The studies of such factors hold great promise for the future of plant biotechnology and plant genetic engineering as they might help in the development of conceptually new techniques and approaches needed today to expand the host range of *Agrobacterium* and to control the transformation process and its outcome during the production of transgenic plants. Here, I review some of the main factors that influence *Agrobacterium*-mediated genetic transformation and discuss their possible roles in this process.

Keywords: *Agrobacterium* strain, antibiotic, explant, light, PGR, temperature, wounding

CONTENTS

INTRODUCTION

Genetic engineering offers a directed method of plant breeding that selectively targets one or a few traits for introduction into the crop plant. The powerful combination of genetic engineering and conventional breeding programs permits useful traits encoded by transgenes to be introduced into commercial crops within an economically viable time frame. The basic idea of plant genetic transformation is the use of genetic engineering techniques to introduce foreign genes into plants. To obtain transgenic plants, several methods have been attempted. These methods include *Agrobacterium* mediated transformation (Gelvin 2003; McCullen and Binns 2006), particle bombardment (Altpeter *et al*. 2005), electroporation (He *et al*. 2001), protoplasts mediated by polyethylene glycol or calcium phosphate (Negrutiu *et al*. 1987; Datta *et al*. 1990) silicon carbide fibers (Frame *et al*. 1994), liposome-mediated transformation (Caboche 1990), and *in planta Agrobacterium*-mediated transformation via vacuum infiltration of whole plants (Bechtold *et al*. 1993). *Agrobacterium*-mediated transformation and particle bombardment are two major methods applied in plants transformation. Compared to particle bombardment, depending on the strain, *Agrobacterium*-mediated transformation integrates lower copy numbers of DNA into the plant genome (Shou *et al*. 2004). Single copy transgenes might tend to be more stably expressed than multiple gene copies (Iglesias *et al*. 1997). Simpler transgene integration patterns and lower transgene copy numbers likely increase the probability of producing a transgenic event that does not exhibit unstable transgene expression due to transgene silencing (Shou *et al*. 2004). Thus, there is increasing adoption of *Agrobacterium*-mediated transformation in both dicot and monocot crops because fewer transgenic events need to be produced. *Agrobacterium*-mediated gene transfer is usually generalized to produce simpler integration patterns, less re-

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arrangements within inserts and reduced problems with co-suppression and instability over generations compared to methods based on direct gene transfer (Zambryski 1988).

The transformation results from the production of a single-stranded copy (T-strand) of transferred DNA (T-DNA) molecule by the bacterial virulence machinery, its transfer into the host cell followed by integration into the host genome (Gelvin 2003; McCullen and Binns 2006; Dodueva *et al*. 2007). The key genes mechanistically involved in T-DNA transfer are the virulence (*vir*) genes of the tumor-inducing (Ti) plasmid. The *vir* genes recognize border sequences harboring the T-DNA regardless of the sequence between the borders (Zhu *et al*. 2000). The T-DNA transfer requires *cis*-acting T-DNA border sequences and the *trans*-acting virulence functions encoded by the Ti plasmid and *Agrobacterium* chromosome (An *et al*. 1988). Therefore, disarmed transformation vectors can be generated from wild-type *Agrobacterium tumefaciens* plasmids by either deleting the complete set of oncogenes or deleting the tumorigenic (oncogene-containing) region from the T-DNA. The genes of interest can be engineered into the resulting binary vector, between the T-DNA borders, and transferred into plant cells. Today *Agrobacterium*-mediated transformation has been used to transform hundreds of species, including economic crops, vegetables, fruit and forest trees, ornamental plants, and medicinal crops.

The *Agrobacterium*-mediated transformation system is influenced by such factors as bacterial strains and cell density, plant species and genotype, plant growth regulators and antibiotics, explant, explant wounding, and light and temperature (Li *et al*. 1997; Salas *et al*. 2001; Zambre *et al*. 2003; Yu *et al*. 2002; Olhoft *et al*. 2003; Shrawat and Lörz 2006; Tzfira *et al*. 2006). The critical point in developing an efficient transformation protocol is to find the right combination of the many factors that act together during transformation. The identification and studies of such factors of these processes hold great promise for the future of plant biotechnology and plant genetic engineering, as they might help in the development of conceptually new techniques and approaches needed today to expand the host range of *Agrobacterium* and to control the transformation process and its outcome during the production of transgenic plants. In this review, some important factors influencing successful *Agrobacterium*-mediated transformation of plants are briefly focused with a discussion of their possible roles in this process.

EXPLANT

A variety of explants can use as target material for *Agrobacterium*-mediated transformation including embryonic cultures, immature embryos, mature seed-derived calluses, meristems, shoot apices, excised leaf blades, root cotyledons, stem segments and callus suspension cultures but the type of explant is also an important fact and it must be suitable for regeneration allowing the recovery of whole transgenic plants. Indeed, it is the totipotency of plant cells that underlies most plant transformation systems.

Several investigators have shown that various tissues, organs, and cell types within a plant may differ in their susceptibility to *Agrobacterium* transformation. Schlappi and Hohn (1992) demonstrated only embryos in which the shoot apical meristem had begun to differentiate showed competence, and the timing of this window differed among the three maize cultivars examined. Ritchie *et al* (1993) showed that in maize, transformation occurred in mesocotyl segments originating from the intercalary meristem region. De Kathen and Jacobsen (1995) showed that only dedifferentiating cells near the vascular system of cotyledon and epicotyl regions of *Pisum sativum* were susceptible to *Agrobacterium* transformation. Cells showing transient expression showed a tendency for a preferential location on the scutellum side near to the place of embryo axis connection, which was also observed by McCormac *et al*. (1998) for intact barley embryos. In sorghum, the source of the explant had a significant effect on the transformation rate (Zhao *et al*. 2000). Immature embryos from field-grown plants showed a higher transformation frequency than immature embryos from glasshouse-grown plants. Embryogenic callus derived from mature seeds has been reported to be the best explant for *Agrobacterium*-mediated transformation in some plant species (Kondo *et al*. 2000; Limanton-Grevet and Jullien 2001; Repellin *et al*. 2001; Suzuki and Nakano 2002; Cheng *et al*. 2003). Almost all studies of *Agrobacterium*-mediated transformation of cereals use tissues consisting of actively dividing embryogenic cells, such as immature embryos and calluses induced from scutella. Immature embryos have been the preferred explants for genetic transformation of cereals, including barley, due to their excellent morphogenetic competence (Cheng *et al*. 2004; Shrawat *et al*. 2006, 2007).

A significant factor that enhances transformation of crop species is dessication of explants prior to, or post, *Agrobacterium* infection. Arencibia *et al*. (1998) reported that air-drying sugarcane suspension cells prior to inoculation slightly improved T-DNA delivery and subsequently increased transformation efficiency. Similarly, air-drying calluses derived from rice suspension cultures the transformation efficiency 10-fold or more as compared to the control without air-drying (Urushibara *et al*. 2001). Cheng *et al*. (2003) reported that desiccation of precultured immature embryos, suspension culture cells, embryonic calluses of wheat, and embrogenic calluses of maize greatly enhanced T-DNA delivery and plant tissue recovery after co-culture, leading to increased stable transformation frequency. This treatment was not only effective in monocot species, but also improved T-DNA delivery in recalcitrant dicot species such as soybean suspension cells based on our preliminary study (Cheng and Fry, 2000). Although the molecular mechanism of desiccation during co-culture remains unclear, it is known that desiccation suppresses the growth of *Agrobacterium.*

Preculture of explants have been shown to influence the transformation efficiency in *Populus nigra* (Confalonieri *et al*. 1994), maize (Ishida *et al.* 1996), wheat (Cheng *et al*. 1997), *Arabidopsis* (Schmidt and Willmitzer. 1998), Chinese cabbage (Zhang *et al*. 2000), canola (Cardoza and Stewart 2003) pepper (Li *et al*. 2003), cotton (Wu *et al*. 2005) kenaf (Herath *et al*. 2005), barley (Shrawat *et al*. 2007). The basis of promotion of *Agrobacterium*-mediated transformation by preculture is not completely understood. Initiation of active cell division upon wounding, improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites and production of v*ir*-inducing compounds by the metabolically active cells are proposed as important contributing factors.

The variation in response of plant tissues to *Agrobacterium* has been attributed in part to differences in the ability of this bacterium to attach to plant cells or to differences in T-DNA transfer machinery (Lippincott *et al*. 1977; Nam *et al*. 1997). It is generally accepted that only plants with an appropriate wound response will develop large populations of wound-adjacent cells that are competent for transformation (Potrykus 1991). However, more significantly, cell death is observed in cultures of many plant cells following exposure to *Agrobacterium*. The modification of transformation parameters can increase the probability of stably transforming some recalcitrant cell types; however, cell death following *Agrobacterium* infection still remains a significant limitation (Gelvin 2003). Tissue browning and necrosis following exposure to *Agrobacterium* occurs in many monocot and dicot plants, including poplar (de Block 1990), grape (Perl *et al*. 1996), sorghum (Carvalho *et al*. 2004; Gao *et al*. 2005), wheat (Parrott *et al*. 2002), tomato, pepper, and lettuce (van der Hoorn *et al*. 2000; Wroblewski *et al*. 2005). *Agrobacterium* transformation triggers expression of many genes in the host cell, including components of plant defense machinery (Ditt *et al*. 2001; Veena *et al*. 2003). On pathogen infection, one of the earliest defense mechanisms activated is the production of reactive oxygen species, referred to as an oxidative burst. The reactive oxygen inter-

mediates produced during the oxidative burst are responsible for activating programmed cell death (Parrott *et al*. 2002). The co-cultivation of *Agrobacterium* with maize or wheat tissues has been reported to result in a process closely analogous to apoptosis in animal cells, where in cell death is characterized by DNA cleavage into oligonucleosomal fragments and defined morphological changes (Hansen 2000). Parrott *et al*. (2002) reported that, after *Agrobacterium* infection, wheat embryos and root cells rapidly produced hydrogen peroxide (H_2O_2), displayed altered cell wall composition and resulted in higher levels of cellular necrosis and subsequent cell death. A correlation between the reduction in cell death and the improved transformation frequency has been demonstrated in rice (Enríquez-Obregón *et al*. 1998), sugarcane (Enríquez-Obregón *et al*. 1997), sorghum (Zhao *et al*. 2000) and maize (Ishida *et al*. 1996). Parrott *et al*. (2002) also observed that lowering the H_2O_2 tension from 7.4 to 2.1 mM significantly reduced the extent of embryo and root cell death in wheat after *Agrobacterium* transformation. It has also been found that *Agrobacterium*-induced necrosis observed in Poaceae can be inhibited by the use of necrosis inhibiting agents, such as silver nitrate (Hansen and Durham 2000). Anti-necrotic treatment of the target tissues may provide an adequate environment for the interaction of *Agrobacterium* with the plant cells by inhibiting necrosis, and may result in increased efficiency of transformation (Enríquez- Obregón *et al*. 1997). One report indicated that maize callus infected with *Agrobacterium* undergoes a rapid, hypersensitive type of cell death and this response was suppressed by expression of two baculovirus genes, p35 and iap (Hansen 2000). However, tissue browning and necrosis after *Agrobacterium* infection are still major obstacles in the genetic transformation of plants and a molecular understanding of cell death via apoptosis-like processes may lead to the minimization of *Agrobacterium*-induced cell death.

EXPLANT WOUNDING

In *Agrobacterium*-mediated transformation systems, physical wounding of explants is commonly done as this greatly influences transformation efficiency (Rashid *et al*. 1996). Plant species may differ in their temporal competence for transformation following wounding. Braun (1947) for first noted this window of competence in *Vinca rosea*. Bacteria were applied to cut plant surfaces various times after wounding. If the plants were inoculated within three days of wounding, tumor induction was relatively efficient. Inoculation four days after wounding resulted in only a few percent of the plants developing tumors, and after five days, tumorigenesis was absent. However, tomato remained susceptible to tumorigenesis up to two weeks after wounding (Braun 1954). Davis *et al* (1991) showed that six days after wounding, tomato plants still retained approximately 25% of the susceptibility of plants inoculated immediately after wounding. Davis *et al.* (1991) also concluded that although suberization of the cell walls, which may present a physical barrier to transformation, did occur four days after wounding, suberized cells still retained a high transformation susceptibility.

The type or method of wounding ranges from simple wounds made during the normal course of explant preparation to particle gun mediated micro-wounding (Bidney *et al*. 1992; Zuker *et al*. 1999). Tissues can also be wounded using *Agrobacterium*-filled syringes which allow some degree of 'delivery' of the bacterium to the target tissue (Chee *et al*. 1989). Another approach is the use of sonication to wound and modify the target tissue to enhance *Agrobacterium* infection. This technique involves subjecting the plant tissue to brief periods of ultrasound in the presence of agrobacteria. The strength of this method is that the cavitation caused by sonication results in thousands of microwounds on and below the surface of the plant tissue. This wounding pattern permits *Agrobacterium* to travel deeper and more completely throughout the tissue than conventional microscopic wounding (**Fig. 1**), increasing the probability of infecting plant cells. In addition, the ultrasound causes micro wounding on the surface and sub-surface layers of the targeted tissue to secret more phenolic compounds, which enhances transformation in soybeans (Santarem *et al*. 1998). Sonication has been used to enhance *Agrobacterium*-mediated transformation of many different plant species (Trick and Finer 1997; Santarem *et al*. 1998: Zaragoza *et al*. 2004; Teixeira da Silva 2005; Flores Solís *et al*. 2007; Pathak and Hamzah 2008).

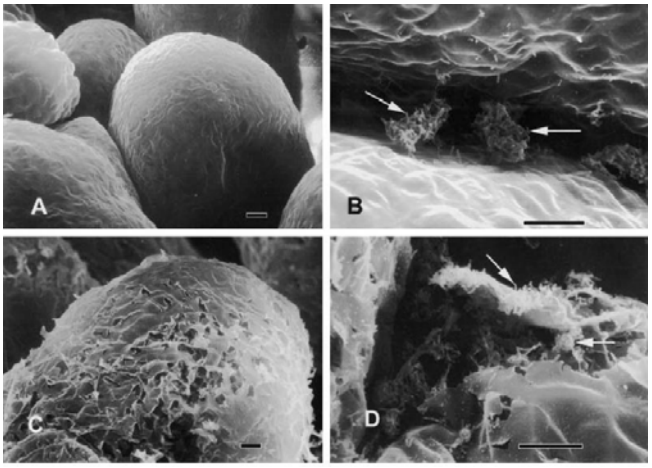

Fig. 1 Scanning electron micrographs of non-sonicated embryogenic suspension tissue (**A** and **B**) and sonicated-treated samples with microwounds caused by sonication visible on the surface (**C** and **D**) of soybean. *Agrobacterium tumefaciens* EHA105 (arrows in **B** and **D**) are clearly evident after 2 days of co-culture and can also be seen colonizing the surface and within the microwounds of the sonicated-treated tissue (**D**). Bar: 10 mm.

Not only does the wound site act as an entry point for bacterium and but also results in the release phenolic substances necessary for *vir* gene activation (Joubert *et al*. 2002). In addition, the increases of transformation efficiency, based on the application of additional phenolic substances, have also been reported in apple (James *et al*. 1993), rice (Aldemita and Hodges 1996), soybean (Santarém *et al*. 1998), cotton (Sunilkumar and Rathore 2001) and barley (Kumlehn *et al*. 2006). However, AS did not enhance the efficiency of transformation in plum (Mannie *et al*. 1991), poplars (Confalonieri *et al*. 1997), and tea (Mondal *et al*. 2001). In monocots, where such compounds are not synthesized, addition of phenolic compounds such as acetosyringone (AS) during plant/bacteria interaction supports the gene transfer (Usami *et al*. 1987; Cheng *et al*. 1997; Hiei *et al*. 1997; Wu *et al*. 2003; Kumlehn *et al*. 2006).

Generally, it is believed that *Vir* gene-inducing signals are released from plants only at wound sites. Phenolics are involved in lignification and healing of the wound, and may also be released from the wound site as antimicrobials (Dixon and Paiva 1995). In one study, two phenolic inducers, AS and a hydroxyacetosyringone, were released in higher amounts from tobacco leaf discs than from unwounded leaves (Stachel *et al*. 1985). However, these compounds were first isolated from cultured plant cells or roots, neither of which had been wounded (Stachel *et al*. 1986). While the cultured cells and tissues used in those studies may not reflect the exudates of intact plants, it nevertheless seems plausible that unwounded plants might release sufficient *vir* gene inducers to stimulate the *vir* regulon. Wounding of plant tissues may also be important to compromise any physical barriers that might block T-DNA transfer. The waxy cuticle that coats plant epidermis may block productive physical contacts between the bacterium and the host cell envelope that are required for T-DNA transfer (Zhu *et al*. 2003). However, leaf mesophyll cells and root epidermal cells are not protected by a cuticle, because they must be permeable to gasses, minerals, and water. Indeed, one study

showed that T-DNA can be transferred to mesophyll cells of unwounded plants (Escudero and Hohn 1997), although transfer in that study occurred only when the bacteria were pre-induced with a synthetic phenolic compound.

It has been proposed that cell division during wound healing may play a role in tumourigenesis (Binns and Thomashow 1988). Dividing cells may have less rigid cell walls that can be more easily breached. In addition, DNA replication may facilitate T-DNA integration, and the dividing cells may be more sensitive to the mitogenic effects of the T-DNA encoded phytohormones. Supporting these ideas is the finding that application of exogenous auxin prior to infection stimulates plant cell transformation (Stover *et al*. 1997; Chateau *et al*. 2000). Other studies reported that *Agrobacterium*-induced tumours were limited to the cambium, which is a meristematic tissue and thus predisposed to cell division (Ghorbel *et al*. 2000). Wounding can cause virtually any plant cell to dedifferentiate and become meristematic (Taiz and Zeiger 2002). This normally occurs during the process of wound healing and is followed by rapid proliferation of cells at the site of injury. Therefore, wounding may be required for differentiated plant cells to begin neoplastic cell division. Wounding may also provide *A. tumefaciens* physical access to internal meristematic tissues such as the cambium (**Fig. 1**).

PLANT SPECIES AND GENOTYPE

The ability of particular *Agrobacterium* strains to transform plant cells is defined by their chromosomal and plasmid genomes which between them must encode all the machinery necessary for attachment and DNA-transfer, but plants produce different inducer molecules that vary in their inducing ability and cellular concentration. This variability leads to differences in the level of *vir* gene expression in different hosts, thereby affecting their sensitivity to infection by *Agrobacterium*. A low level of *vir* gene expression can make a plant recalcitrant by virtue of the inability of the bacterium to synthesize and transfer sufficient T-strand DNA essential for a successful infection.

Various plant species differ greatly in their susceptibility to infection by *Agrobacterium* (Anderson and Moore 1979; Porter 1991; Cheng *et al*. 2004). Even within a species, different cultivars or ecotypes may show vastly different degrees of usceptibility to tumorigenesis by particular *Agrobacterium* strains. These differences have been noted in maize (Ritchie *et al*. 1993), various legumes (Hood *et al*. 1987; Owens and Cress 1984), aspen (Beneddra *et al*. 1996), *Pinus* species (Bergmann and Stomp 1992), tomato (van Roekel *et al*. 1993), *Arabidopsis* (Nam *et al*. 1997), grape (Lowe and Krul 1991), and other species. *Agrobacterium*-mediated transformation of higher plants is well-established for dicotyledonous species. In recent years the frequency of gene transfer to monocotyledonous species has been greatly improved. although frequency of gene transfer to monocotyledonous species has been greatly improved and successful *Agrobacterium*-mediated transformation has been reported in rice (Hiei *et al*. 1994, 1997; Toki *et al*. 1997), maize (Ishida *et al*. 1996), barley (Tingay *et al*. 1997; Shrawat *et al*. 2007), wheat (Cheng *et al*. 1997) and sorghum (Zhao *et al*. 2000; Carlos *et al*. 2004; Carvalho *et al* 2004), the difference in the competence of *Agrobacterium* to infect genotype or species has also been a major drawback in the genetic transformation of elite cultivars of monocotyledonous, especially in extending the host range to commercial cultivated cultivars. Amongst the cereals transformed to date, rice appears to be the least genotype dependent, as more than 40 genotypes of *japonica*, *indica* and *javonica* have been transformed. In comparison, only a few model genotypes have been successfully used in the *Agrobacterium*-mediated transformation of other major cereal crops: for example, cultivar A188 or its hybrids in maize, cv. 'Bobwhite' in wheat, 'Golden Promise' and 'Igri' in barley, and 'Ja 60-5' in sugarcane. Although transgenic plants have recently been recovered from elite cultivars or lines of sorghum (Zhao *et al*. 2000), maize (Gordon-Kamm *et al*. 2002) and barley (Wang *et al*. 2001), the overall transformation frequency is lower than that with model cultivars. These studies indicate that the genotype-dependent response in *Agrobacterium*-mediated transformation of cereals is a major drawback in extending *Agrobacterium*-mediated transformation systems to elite cultivars of economically important cereals. Therefore, it becomes important to make elite cultivars amenable to tissue culture and to improve their regenerability by manipulating existing tissue culture medium.

The difference in the susceptibility of genotypes to *Agrobacterium* may be a result of the presence of inhibitors of the *Agrobacterium* sensory machinery. 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA), the major organic exudate of maize seedling roots, specifically inhibits induction of *vir* gene expression by an as-yet-unknown mechanism (Zhang *et al*. 2000; Maresh *et al*. 2006). The relative difference in the resistance of agronomically important plant species, to *Agrobacterium*-mediated genetic transformation may be a result of the presence of such inhibitors, rather than to insufficient activation of the *Agrobacterium* virulence machinery by host cell exudates. It seems that naturally occurring inhibitors directed against signal perception by the *VirA*/*VirG* two-component regulatory system may play an important role in host defense (Zhang *et al*. 2000). MDIBOA is not the only natural inhibitor of *vir* gene induction that. The indoleacetic acid was also shown to inhibit *vir* gene induction (Liu and Nester 2006). However, plants may perceive *Agrobacterium* and the transferred transgenes as foreign invaders and use their defense systems to battle the infection process and expression of foreign genes.

ANTIBIOTICS

For transformation, plant tissues are infected by co-cultivation with a disarmed *A. tumefaciens* carrying a gene of interest in an antibiotic-free medium for 2–3 days. After co-cultivation, the bacterium needs to be suppressed so as not to interfere with the growth and development of the transformed plant cells (**Fig. 2**). Successful transformation using *Agrobacterium* depends not only on the efficiency of the plant regeneration systems but also on the subsequent elimination of this bacterium from transformed cells. The elimination of *Agrobacterium* is usually achieved by adding one or more antibiotics to the culture medium and is quite important because the continued presence of *Agrobacterium* can present a problem for identifying transformants or interfere with the growth and development of the transformed plant cells or cause the death of the cultures (Horsch *et al*. 1985; Matzk *et al*. 1996). Carbenicillin and cefatoxime are the most commonly used antibiotics for this purpose.

In carnation (Song *et al*. 2005) and barley (Shrawat *et al*. 2007) have been shown that infection by *Agrobacterium* increased the resistance of leaf explants to selective agents. It is well-known that plants have different defense mechanisms to abiotic and biological stresses upon attack. These mechanisms can be physical defenses including the cell wall components such as lignin and cellulose, chemical defenses such as tannins and phenolics and other defense mechanisms involving inducible components that are deployed only when they are needed (Muthukrishan *et al*. 2001). In the case of inducible defense mechanisms, different inducible compounds are produced upon challenge inoculation with a pathogen. Some novel proteins are among these compounds and are collectively called pathogenesis-related proteins (PRs) (van Loon and van Strien 1999). Induction of PRs has been found in many plant species. The increased resistance to the selective agents upon agrobacterial infection may be related to the induction of PRs in the leaf explants.

In plant transformation, it is difficult to choose the right concentration of selective agents to enable organogenesis on one hand and to avoid escapes on another hand. Antibio-

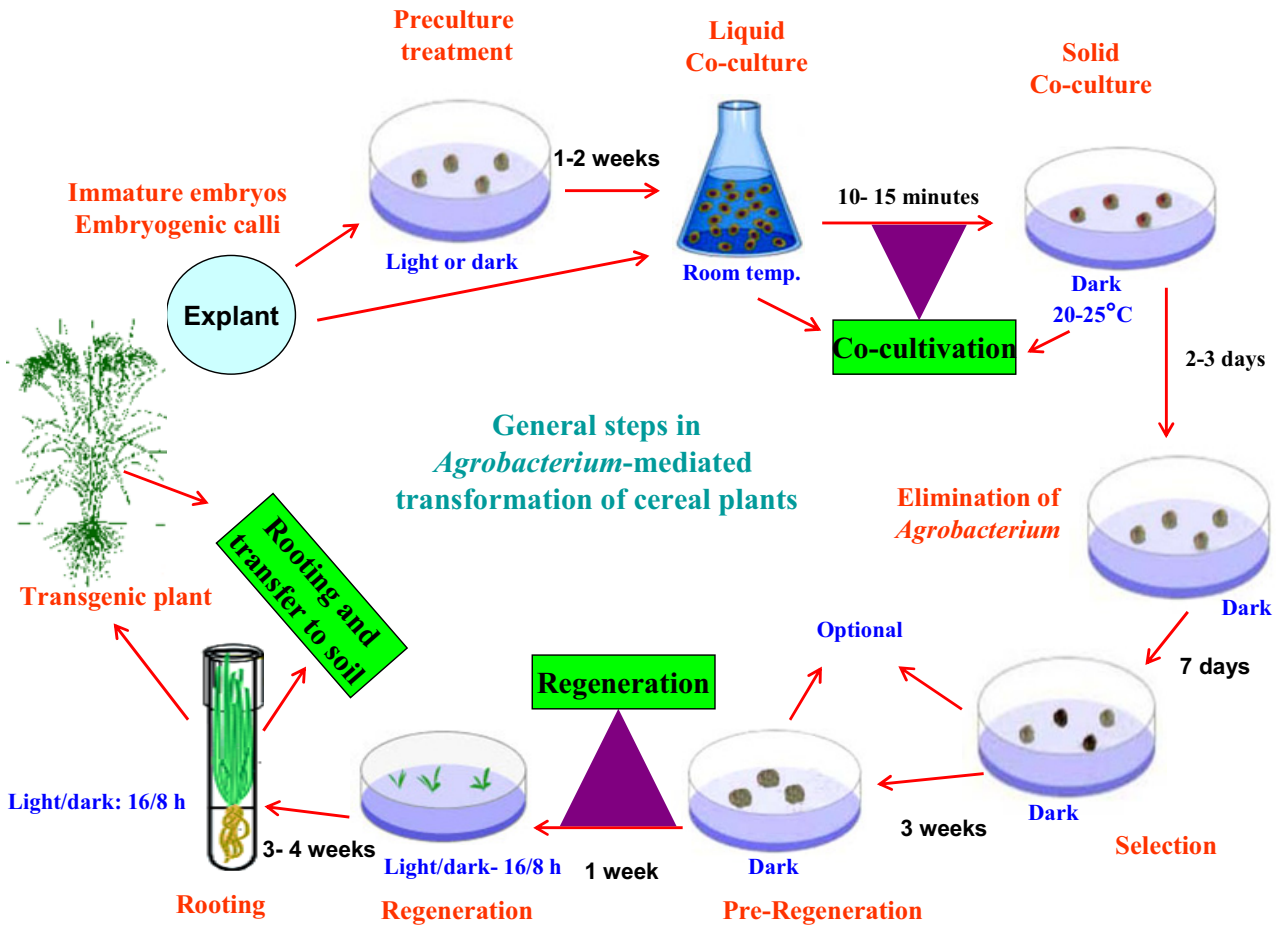

Fig. 2 General scheme for *Agrobacterium*-mediated transformation of cereal plants.

tics may inhibit cell wall synthesis (Holford and Newbury 1992). Contamination reduces regeneration rates and induces plant death (Shackelford and Chlan 1996; Hammerschlag *et al*. 1997; Ling *et al*. 1998; Estopa *et al*. 2001). This effect could lead to decreased transformation efficiency. Therefore, it is critical to have the correct ration of antibiotics to achieve antibiotic protection/selection and adequate rates of organogenesis. There have been many reports concerning the toxicity of antibiotics to callus growth and shoot regeneration (e.g. Teixeira da Silva and Fukai 2004), but only a few on their toxicity to somatic embryogenesis (Eapen and George 1990; Nakano and Mii 1993; Sarma *et al*. 1995; Tang *et al*. 2000). Although they can be a guide to the effects on somatic embryogenesis, a better understanding of the toxicity of antibiotics to both *Agrobacterium* and somatic embryos is still necessary, particularly for those species in which somatic embryos are the tissue capable of regenerating plants following transformation.

The use of antibiotics for eliminating *Agrobacterium* is limited by the fact that some antibiotics, such as carbenicillin and ticarcillin, can be inactivated by f3-lactamases produced by bacteria while others, such as cefotaxime, are highly resistant to f3-lactamases but inhibit plant regeneration (Shackelford and Chlan 1996; Hammerschlag *et al*. 1997; Ling *et al*. 1998). There are, however, several reports that another antibiotic timentin (ticarcillin coupled with a specific f3-lactamase inhibitor clavulanic acid) can efficiently eliminate *Agrobacterium* without inhibiting plant regeneration (Chevreau *et al*. 1997; Cheng *et al*. 1998; Ling *et al*. 1998; Tang *et al*. 2000).

PLANT GROWTH REGULATORS

Plant growth regulators (PGRs) are essential in plant transformation. The choice of PGR is the most important single factor affecting transformation efficiencies measured as frequencies of transient expression and stable integration. Competence for transformation may either be absent or low in recalcitrant explants; however, it can be enhanced by PGR treatments (Valvekens *et al*. 1988; Sangwan *et al*. 1992; Geier and Sangwan 1996; Villemont *et al*. 1997). An explant becomes susceptible to *Agrobacterium* when it is when pre-cultured on medium containing PGRs (Valvekens *et al*. 1988; Potrykus 1990; Sangwan *et al*. 1992; Chateau *et al*. 2000). In many other monocots, 2,4-D-derived calluses and the presence of 2,4-D in the cocultivation medium enhances transformation efficiency (Rashid *et al*. 1996; Wu *et al*. 2003). In *Typha latifolia* a significantly higher percentage of calluses generated using picloram showed transient GUS activity (Nandakumar *et al*. 2004). In barley dicamba in the callus induction and maintenance media generally promoted transient expression and subsequent stable transformation (Trifonova *et al* 2001). In kenaf, pre-culturing the explants for 2 days in benzyl adenine containing medium, was found to enhance the transient GUS expression (Herath *et al*. 2005).

PGR treatment activates cell division and dedifferentiation in many tissues. The stimulation of cell division by PGRs suggests that efficient *Agrobacterium* transformation may occur at a particular stage of the plant cell cycle (Chateau *et al*. 2000). Villemont *et al*. (1997) investigated the role of the plant cell cycle in *Agrobacterium*-mediated transformation of *Petunia* mesophyll cells that had been synchronized with cell cycle phase-specific inhibitors. Non cycling cells that had not been treated with PGRs, could not be transformed either transiently or stably to express a T-DNA-encoded *gus*A transgene. Cells treated with mimosine, which blocks the cell cycle in late G1 phase, similarly could not be transformed. In addition, the cycling cells that showed the highest transformation competence were those cells that

showed a very high S and G2 phase: M phase ratio. The authors concluded that T-DNA could be taken up, translocated to the nucleus, and expressed in cells conducting DNA synthesis but in the absence of cell division, and thus that *Agrobacterium*-mediated transient transformation required S phase DNA synthesis. Subsequent cell division was required for T-DNA integration and stabilization of transformation. The development of a direct assay for T-strand uptake and nuclear translocation that does not depend upon T-DNA-encoded gene expression is required to resolve these two alternatives.

LIGHT

Damgaard and Rasmussen (1991) reported increased transformation rates in *Brassica* plants with *A. rhizogenes* under continuous illumination. Escudero and Hohn (1997) reported inhibition of T-DNA transfer in intact tobacco seedlings sprayed with *A. tumefaciens* and kept in the dark. Clercq *et al*. (2002) found that 24 hours continuous darkness inhibited *Agrobacterium*-mediated transformation in bean compared to light for 16 h per day. However, light is associated with a number of physiological factors, such as plant hormone levels, cell proliferation, cell cycle stage (Villemont *et al*. 1997; Zambre *et al*. 2003). However, light is associated with a number of physiological factors, such as plant hormone levels, cell proliferation, cell cycle stage (Villemont *et al*. 1997; Zambre *et al*. 2003). Whether the promotive effects of light described here are mediated by any of these physiological factors remains to be determined. On the other hand, the efficiency of T-DNA transfer depends largely on how efficiently *vir* genes are induced. In this respect, light has been shown to enhance the amount of the phenolic *vir* gene inducer from the orchid *Dendrobium* (Nan *et al*. 1997). Transient expression of a CaMV p35S-*uid*A gene delivered to embryos or seedlings of *Picea* by particle bombardment was not affected by light conditions (Ellis *et al*. 1991; Gray-Mitsumune *et al*. 1996). Thus, the stimulatory effect of light may be specific for *Agrobacterium*-mediated T-DNA delivery.

Many *Agrobacterium*-based plant transformation protocols for crop and model plant species use dark co-culture conditions without specifying the effect on transformation rate. Incubation in darkness seems to improve the morphogenic capacity of callus or explants (Mohamed *et al*. 1992; Compton 1999), essentially by preserving endogenous light-sensitive hormones (Pádua *et al*. 1998; Compton 1999) or by preventing accumulation of phenolic compounds (Arezki *et al*. 2001). Compton *et al*. (1999) found that dark pretreatment improved subsequent shoot regeneration from cotyledonary node explants. In carnation, constant darkness during coculture was reported to increase transformation (Zuker *et al*. 1999). In *Typha latifolia*, higher transient activity occurred in dark cultured callus rather than in light cultured callus (Nandakumar *et al*. 2004). Dark pretreatment lead to less vascular tissue and thinner cell walls (Hartmann 1997), so the penetration of *Agrobacteria* may be enhanced. Compton (1999) hypothesized that incubation in darkness improved the morphogenic capacity of callus or explants.

Some studies showed that the effect of light on *Agrobacterium*-mediated transformation was mainly derived from photoperiod. Zambre *et al*. (2003) reported that light strongly promoted gene transfer from *A. tumefaciens* to plant cells, and even the transgenic frequency in co-cultivation under continuous light was higher than in co-cultivation under a 16 h light/18 h dark regime. In *Arabidopsis uid*A expression correlated highly and positively with the light period used during co-culture; it was severely inhibited by darkness and enhanced more under continuous light than under a 16 h light/8 h dark photoperiod (Zambre *et al*. 2003).The disparity in results from different studies of light effects on transformation might be due to the interaction between light regime and other factors.

TEMPERATURE

Temperature has been considered a factor affecting the capacity of *Agrobacterium* to transfer the T-DNA to plant cells. Early studies on *A. tumefaciens*-mediated tumorigenesis showed that high temperatures were detrimental for tumor development (Braun 1958). Jin *et al*. (1993) reported that the suppression of tumor development was due to a conformational change in *vir*A produced at high temperatures of about 32°C. Bacterial conjugation studies in which an incompatibility group, Q plasmid, was mobilized by the T-DNA transfer machinery showed that 19°C was the optimal temperature for transfer (Fullner and Nester 1996). The temperature of co-cultivation of *Agrobacterium* and soybean explants was shown to be critical by Kudirka *et al*. (1986). At 30°C co-cultivation for 48 h, transformation was suppressed, whereas at 25°C co-cultivation transformation was successful. If the heat treatment followed the 25°C co-cultivation, transformation was only reduced, demonstrating that thermosensitivity was highest during the T-DNA transfer from *Agrobacterium* to the host plant cell. Dillen *et al*. (1997) investigated temperature effects on gene transfer to plants using callus from *Phaseolus acutifolius* and leaf discs from *Nicotiana tabacum*. The GUS reporter gene was used to detect transient expression, soon after cocultivation. The optimal temperature for gene transfer in both species was 22°C and there was no difference between 19 and 22°C for tobacco. They reported a dramatic decrease in transient GUS expression when the temperature increased from 22°C to 25°C. Srivatanakul *et al*. (2001) evaluated temperature effects on kenaf (*Hibiscus cannabinus*) shoot apex survival using *Agrobacterium*-mediated transformation. In contrast to the results of Dillen *et al*. (1997), they reported that temperatures of 25 and 28°C yielded significantly greater kenaf shoot apex survival on selection medium than 16 and 19°C. In cotton, co-cultivation with *Agrobacterium* at a lower temperature significantly improved transformation efficiency (Jin *et al*. 2005). Kondo *et al*. (2000) tested the effect of four temperatures, namely 18, 20, 22, and 24°C on T-DNA delivery with garlic calluses. The highest transient GUS expression was observed at 22°C, in which 64% of the total calluses showed GUS activity. The ratio of GUS-stained calluses to total calluses decreased by 85% at 20°C and by 69% at 24°C. Sales *et al*. (2001) have also reported the influence of temperature during co-cultivation period on transient and stable T-DNA integration. Out of four different temperatures (15, 19, 25 and 32°C) used for co-cultivation of target tissues; they found that the highest number of stable transformed plants occurred at 25°C. Higher transformation frequency was observed in maize immature embryo transformation at 20°C than at 23°C when using a standard binary vector (Frame *et al*. 2002). Transgenic maize plants have also been obtained from elite inbred lines PHP38 and PHN46 by co-culture of the immature embryos at 20°C followed by 28°C subculture (Gordon-Kamm *et al*. 2002). These results indicate that the optimal temperature for T-DNA delivery may not be optimal for stable transformation with a given species and explant. The optimal temperature for stable transformation should be evaluated with each specific explant and *Agrobacterium* strain involved.

AGROBACTERIUM STRAIN

Of various factors influencing the frequency of *Agrobacterium*-mediated transformation, one of the most important is the infecting ability of the *Agrobacterium* strain. A large number of strains of *A. tumefaciens* have been isolated, such as Ach5, C58, and Bo542, and the host range was characterized (Hooykaas 1984; Hood *et al*. 1987). It was also found that the infecting ability and range was dependent upon the Ti plasmid (Hood *et al*. 1993). Strain A281 is a super-virulent strain developed from Bo542, harboring pTiBo542 (Watson *et al*. 1975). A disarmed pTiBo542 was constructed by replacing the nature T-region with a kanamycin resistance gene (Hood *et al*. 1986). Both EHA101

and EHA105, harboring the disarmed pTiBo542, are identified to be powerful in plant transformation (Hood *et al*. 1993). Studies have demonstrated improved efficiency of T-DNA delivery to monocotyledons cells by use of 'super-virulent' strains or 'super-binary' vectors (Hiei *et al*. 1994; Ishida *et al*. 1996; Tingay *et al*. 1997; Zhao *et al*. 2000; Adelina *et al*. 2001).

The difference in the competence of *Agrobacterium* to infect genotype or species has also been a major drawback in the genetic transformation of elite cultivars of cereals. For example, when McCormac *et al*. (1998) compared the T-DNA transfer efficiency in wheat between two *A. rhizogenes* strains (LBA9402 and Ar2626) and two *A. tumefaciens* strains (LBA4404 and EHA101), they found that only EHA101 facilitated T-DNA delivery successfully into wheat. In the majority of cereals, genetic transformation has been achieved using *Agrobacterium* strains LBA4404, EHA101 and their derivatives (EHA105 from EHA101, AGL0 and AGL1 from EHA101) (Cheng *et al.* 2004). Huang and Wei (2006) have been shown that EHA105 is superior to LBA4404 in transformation of maize. EHA105 has also been found to be more suitable for transformation of other cereals (Rashid *et al*. 1996). The hypervirulence of EHA105 derives from the disarmed pTiBo542 (Hood *et al*. 1993), in which the *virG* and the *virA* genes increase the induction of the *vir* genes, necessary for T-DNA transfer (Gelvin 2000, 2003). Thus, the improved plant transformation efficiency observed with EHA105 is probably related to the increased induction of the vir genes. Chabaud *et al*. (2003) were evaluated *A. tumefaciens* transformation of the model legume *Medicago truncatula* cv. 'Jemalong' (genotype 2HA) for strains LBA 4404, C58pMP90, C58pGV2260 and AGL1. The highest transformation efficiency was obtained with the disarmed hypervirulent strain AGL1. Similar results have been obtained for other species, such as alfalfa with the hypervirulent strain A281 (Chabaud *et al*. 1988) and rubber tree with AGL1 (Montoro *et al*. 2000). The hypervirulence of AGL1 derives from the disarmed pTiBo542, in which the *virG* and the virA genes increase the induction of the vir genes (Turk *et al*. 1991), necessary for T-DNA transfer (Gelvin 2000). Thus, the improved plant transformation efficiency observed with AGL1 is probably related to the increased induction of the *vir* genes.

The combination of a standard binary vector in a supervirulent strain and a superbinary vector in a regular strain has resulted in the successful transformation of rice (Hiei *et al*. 1994). As a result of the success in rice, identical or similar combinations were used for the genetic transformation of maize (Ishida *et al*. 1996; Negrotto *et al*. 2000; Zhao *et al*. 2001), barley (Tingay *et al*. 1997; Wu *et al*. 1998), sorghum (Zhao *et al*. 2000), wheat (Khanna and Daggard 2002) and sugarcane (Arencibia *et al*. 1998). With some crops, such as maize and sorghum, an efficient transformation system was established only with super-binary vectors in LBA4404, whereas a standard binary vector in a supervirulent strain showed low transformation frequency even with improved co-culture conditions in maize (Frame *et al.* 2002). Ishida *et al*. (1996) achieved high efficiency transformation of A188 with a super-binary vector in *Agrobacterium*, although the frequencies in hybrids between A188 and other genotypes were lower. Zhao *et al*. (2001) have been developed a high throughput transformation system in Hi-II with *Agrobacterium* LBA4404 harboring the 'Super-binary' vector. Huang and Wei (2006) have been shown that EHA105 is superior to LBA4404 in transformation of maize. A comparison of different *Agrobacterium* strains demonstrated that AGL0, a hypervirulent strain containing a disarmed pTiBo542 plasmid, was better at generating wheat transformants than other strains tested (Weir *et al*., 2001) The weakly virulent *Agrobacterium* strain LBA4404, was successful in transforming wheat only when augmented by the superbinary plasmid pHK21 which possessed extra copies of *vir* B, C and G genes from pTiBo542 but not when carrying a standard binary plasmid (Khanna and Daggard 2003). Further evidence of the positive effect of additional *vir* genes was provided by the demonstration that a 15 Kb fragment of pTiBo542 on a pSOUP helper plasmid enhanced TDNA delivery and the production of transgenic wheat plants, even when in a hypervirulent AGL1 background already containing pTiBo542 as a resident Ti plasmid (Wu *et al*. 2003). Zhangsun *et al*. (2007) have been shown that co-cultivation of sugarcane calli with *A. tumefaciens* carrying super-binary vectors resulted in efficient recovery of transgenic plants. In this research, both EHA105 and A281 provided successful results. The best was EHA105, while LBA4404 did not provided satisfactory results in comparison with the other two strains.

It has also been shown that the inclusion of the constitutively active *vir*G mutant gene in a binary vector increases T-DNA delivery in both monocot and dicot species (Hansen *et al*. 1994; Wenck *et al*. 1999; van der Fits *et al*. 2000; Ke *et al.* 2001). These studies indicate that the use of other strains with a combination of the super-binary or binary vectors containing a constitutively active *virG* gene may further improve the transformation efficiency in recalcitrant crops. The use of various other strains and the combination with superbinary vectors or binary vectors with a constitutively active *virG* may further improve transformation efficiency in many or all monocot species.

AGROBACTERIUM CELL DENSITY

Agrobacterium cell density is known to directly affect the efficiency of transformation. Once the plant tissue is saturated, increasing inoculum density will not produce further increases. In addition, excessively high *Agrobacterium* density may damage explants because the infection of *A. tumefaciens* could result in extensive enzymatic browning and cell death (Olhoft *et al*. 2003). However, the optimal cell densities may also depend on some other factors, such as *Agrobacterium* strain, *Agrobacterium* cell viability, plant species, and the tissue used. For example, Amoah *et al*. (2001) reported that increasing inoculum density (OD_{600}) from 1.0 to 1.5 for *Agrobacterium* AGL1 caused substantial increases in the number of explants producing spots in wheat transformation. Mondal *et al*. (2001) indicated that the optimal inoculum density for both *Agrobacterium* EHA105 and LBA 4404 was OD_{600} = 0.6 in tea. Kumria *et al*. (2001) reported that an OD_{600} in the range of 0.3–0.4 for pTOK233 and 0.4–0.6 for pJB90GI was optimal for transformation and regeneration in rice. In cotton, an overgrowth of bacterium resulted in a high frequency of contamination and consequently decreased plant regeneration (Jin *et al*. 2005). Therefore, considering multi-factors affecting transformation, investigating the appropriate *Agrobacterium* inoculum density for a specific transformation system is essential.

PERSPECTIVES AND ADVANCES IN *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Owing to the predominant role of plant crops in the human diet, food security in the future can not be achieved without major increases in plant production. Therefore, in recent years, genetic transformation of plants has become an important tool for cultivar improvement with desirable traits. Despite tremendous successes in genetic transformation of plant crops, one of the major technical challenges facing plant transformation is the development of methods to produce a high proportion of plants routinely showing stable and precise transgene expression.

In less than 30 years, the use of *Agrobacterium* to genetically transform plants has advanced from a dream to a reality. Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily. In some developed countries, a high percentage of the acreage of such economically important crops as corn, soybeans, cotton,

canola, potatoes, and tomatoes is transgenic; an increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium*-mediated transformation. However, there remain many challenges. Many economically important plant species, or elite varieties of particular species, remain recalcitrant to *Agrobacterium*-mediated transformation. Tissue browning and necrosis following *Agrobacterium* infection is still a major obstacle in genetic transformation of cereals. Although, efficient transformation systems have been established in some crop plants and several important factors influencing *Agrobacterium*-mediated transformation of plant have been investigated and optimized, development and improvement the transformation parameter such as optimizing inoculation, co-culture condition, extending the range of transformable genotypes and use of readily available explants are still needed in many plant species.

A better understanding of the host biological processes involved in transformation will unravel principles that govern *Agrobacterium*–host cell interactions which result in the unique event of *trans*-kingdom gene transfer, afford novel insights into the cellular processes themselves, and help develop new strategies for efficient genetic manipulation of plant. Recently, the identification and molecular characterization of the plant genes involved for successful *Agrobacterium*-mediated transformation have opened up new avenues for better understanding of the plant response to *Agrobacterium* infection. Such information may help to develop methods to enhance the transformation frequency of economically important plant species.

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