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Agrobacterium-Mediated Plant Transformation under *in Planta* Conditions

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ABSTRACT

This review describes methods of *Agrobacterium*-mediated T-DNA transfer to plant vegetative and generative cells under *in planta*, *ex planta* and "floral dip" conditions, including designing and testing methods that require the *in vitro* cultivation of transgenic plant cells and tissues. At present, most methods of *Agrobacterium*-mediated transformation are based on the coincubation of plant vegetative organs and tissues (leaves, roots, stems, or meristems) with bacterial cell suspensions. Adult plants are then regenerated from the cultivated cells or tissues. This approach gives rise to chimeric transformants and bottlenecks with the regeneration from the cultivated cells or tissues of some monocotyledonous plants. Alternatively, T-DNA integrates into the plant genome as a result of treatment of the male and female plant gametophytes with *Agrobacterium* cells containing activated *vir* genes by using the "floral dip" method and its variations. Since the transformation frequency is not sufficiently high, especially for monocotyledonous plants, factors affecting the transformation frequency and the *Agrobacterium*-mediated T-DNA transfer mechanism have been analyzed.

Keywords: T-DNA, methods, transfer mechanism, virulence proteins
Abbreviations: AS, acetosyringone

CONTENTS

INTRODUCTION

Agrobacterium elicits crown-gall tumors on most di- and monocotyledonous plant species. The formation of tumors occurs after transfer of a portion of single-stranded DNA (T-DNA, [transferred DNA]) as part of the Ti-plasmid into the host-cell genome. The discovery of stable integration of the agrobacterial T-DNA into the plant genome was established three decades ago (Chilton *et al*. 1977). Transformation using the *Agrobacterium tumefaciens* T-DNA transfer system is still a prevalent method for generating transgenic plants, since any genes placed between the T-DNA borders can be transferred to the host cell. In addition to *A. tumefaciens*-mediated transformation, particle-gun-based methods using greenhouse plants are also available but are not considered in this review.

The T-DNA molecule itself does not encode protein machinery for its transport from the agrobacterial cell into the host cell genome, since these functions are coded by the bacterial *vir* genes located on the Ti-plasmid, the agrobacterial chromosomal locus (*chv*) (for reviews, see Chumakov 2001; Gelvin 2003; Tzfira and Citovsky 2003), and some host-cell partner genes (Gelvin 2003; Hwang and Gelvin 2004).

The *chv* locus is expressed constitutively, whereas the *vir* locus (ten operons, *vir*A–J) is induced by plant metabolites such as the phenolic cell-wall-synthesis precursors acetosyringone (AS) and hydroxyacetosyringone, which begin to be synthesized by plant cells after damage or during growth or regeneration of plant tissue (Stachel *et al*. 1985, 1986a). Wounding allows the bacteria to penetrate directly to specific plant receptors, since removal of a physical obstacle (the cell wall) exposes specific receptor sites to the bacteria by enhancing the contact of virulent bacteria in tissues susceptible to infection (Lippincott *et al*. 1977).

Besides the production of AS, cell division and/or DNA synthesis are also important for plant cell competence (susceptibility to agrobacterial infection) (Binns and Thoma-

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show 1988). In addition, the wound sap has lower pH values and contains sugars and amino acids, which also may induce *vir* genes, though to a lesser extent (Ankenbauer and Nester 1990; Cangelosi *et al*. 1990).

The single-stranded (ss) non-polar linear molecule of T-DNA (the T-strand) is produced with the involvement of the AS-activated *vir* genes (Stachel *et al*. 1986a, 1986b, 1987). The products of the AS-induced genes *vir*D1 and *vir*D2 (endonucleases VirD1 and VirD2) stimulate excision of T-DNA; then the T-strand, piloted by VirD2, is transferred into the plant-cell cytoplasm, where a T-complex is formed after the T-strand gets coated with VirE2 (Citovsky *et al*. 1989; Rossi *et al*. 1996; Tinland 1996). VirD2, responsible for the T-complex piloting from the bacterial cell to the plant-cell nucleus, binds to the 5' end of T-DNA. VirD2 is also responsible for recognition of the nuclear pore and the insertion of T-DNA into the plant chromosome (Koulikova-Nicola *et al*. 1993; Pansegrau and Lanka 1996; Citovsky *et al*. 1994; Tinland 1996).

T-DNA is transferred into the plant cell cytoplasm as a DNA–protein complex (the T-complex) that consists of T-DNA, with the protein VirD2 attached to its 5′ end, and the protein VirE2, interacting with ssT-DNA (Citovsky *et al*. 1989; Volohina *et al*. 2005; Volohina and Chumakov 2007). The ssT-DNA–VirD2–VirE2 complex is transferred to the plant cell independently, the export of VirE2 is dependent on the presence of the protein VirE1 in the bacterial cell, and, possibly, the VirE2–VirE1 complex is transferred via a VirB-independent channel (Chen *et al*. 2000). A recent study demonstrated the capacity of VirE2 for interacting with a planar lipid membrane and for forming a channel in it that opens upon application of an electric field of 100 mV and can allow the passage of short oligonucleotides (Dumas *et al*. 2001; Duckely and Hohn 2003; Chumakov *et al*. 2004). How the VirE2-mediated transfer of T-DNA across artificial and natural membranes occurs is not known, however.

The precise mechanism of T-DNA transfer from bacterial to plant cells has not been detailed for some steps (T-DNA transfer across the plant-cell wall and membrane and T-complex transportation within the plant-cell cytoplasm), and it is necessary to understand it if we are to improve agrobacterial transformation technologies.

T-DNA TRANSFER FROM AGROBACTERIAL TO PLANT CELLS: EXCRETION AND UPTAKE OF T-DNA OR TRANSFER DURING CONTACT?

The analogy between the T-DNA transport mechanism and the export of proteins considered as a new paradigm is the fourth-type transfer of the protein–DNA complex from donor to recipient cells (Christie 1997). A possible way of DNA penetration into the recipient cell after its release from the donor cell is its transfer across the pores in the recipient membrane (Dumas *et al*. 2001; Duckely and Hohn 2003; Chumakov *et al*. 2004). In this case, conjugation can be considered to be a special case of transformation. However, ssT-DNA transfer via a VirE2-dependent pore across the artificial or natural membrane has not been observed. An alternative hypothesis holds that T-DNA is absorbed by the plant cell during endocytosis through a cytoskeletal mechanism (Chumakov 2001; Chumakov *et al*. 2002).

It is well known that the *Agrobacterium* infection of plants occurs at wound sites of plant cells, but it is unknown which way is used by *A. tumefaciens* for ssT-DNA transfer across the unwounded cell wall into the plant cell. Krens *et al*. (1985) proposed that the forming primary cell wall of tobacco protoplasts is responsible for the attachment of pathogenic agrobacteria. Several years later, an elongation factor (EF-1α) homologous to vitronectin was found in the plant cell wall (Zhu *et al*. 1994). It is of interest that vitronectin-like protein can serve as a receptor in agrobacterial attachment (Wagner and Matthysse 1992).

Later, it was shown that *Agrobacterium* attachment as such is not necessary for transformation (Escudero and Hohn 1997). However, Gelvin with coworkers identified more than 70 *Arabidopsis* T-DNA insertion (including attachment-defective) mutants (*rat* mutants) that are resistant to *Agrobacterium* transformation (Nam *et al*. 1999). Using a two-hybrid yeast system and a processed, but not cyclized, form of VirB2 as a bait protein, Gelvin (Gelvin 2003; Hwang and Gelvin 2004) identified two classes of *Arabidopsis* proteins that strongly and specifically interact with this major T-pilus constituent. It seems that three BTI proteins localize at the periphery of root cells in transgenic *Arabidopsis* plants and are involved in the initial interaction of *Agrobacterium* with plant cells, suggesting that BTI proteins may contact the *Agrobacterium* T-pilus (Hwang and Gelvin 2004). But no significant difference in the attachment of *A. tumefaciens* C58 pretreated with acetosyringone at 19°C (T-pilus-favorable) and 30°C (T-pilus-blocking) to wheat-root-hair tips was observed (Kalaptur *et al*. 2004).

The involvement of VirB proteins in the conjugative contact and pilus (T-pili) formation was first suggested by Engstrom *et al*. (1987). The agrobacterial pili were first visualized and their participation in the conjugative transfer of plasmid pML122 was shown by Fullner *et al*. (1996). The T-pilus is essential for *Agrobacterium*-mediated transformation; however, it is not clear whether pili take part only in the initial contact and drawing together of the membranes, or they are involved in transfer of genetic information.

A 2-h incubation with AS induces the formation of extracellular proteinaceous structures resembling the T-pili in *A. tumefaciens* strain C58 (with a Ti-plasmid) but not in strain LBA288 (without it). No differences were observed in the wheat root hair attachment of *A. tumefaciens* Ti-plasmid-harboring strain and strain LBA288 lacking the Ti plasmid after AS treatments (Kalaptur *et al*. 2004).

It is believed that agrobacteria can carry adhesive molecules or a cell-wall-degrading enzyme at the contact site, which provides for polar attachment. The inducible virulence protein VirB1 can be proposed as such an enzyme; it has β-glycosidase activity, it is anchored to the outer membrane, and its C-terminal region (VirB1*) can be exposed outside (Baron *et al*. 1997). Agrobacteria can form aggregates or short pilus-like structures composed of VirB1* protein at one of the cellular poles (Chumakov and Kurbanova 1998). It is not improbable that the polar attachment allows agrobacteria to degrade the plant cell wall.

Silverman (1997) proposed that DNA is transferred by its excretion and propilin transport inside the pilus channel to the pilus end. Also intriguing is the question whether the membrane fusion after the contact and the information transfer is carried out by means of the fusion pore or whether T-DNA is transferred through the bacterial-membrane pore formed by the VirE2 proteins, as suggested by Dumas *et al*. (2001). VirE2 also interacts in yeast with several of the *Arabidopsis* importin-proteins, suggesting that VirD2 and VirE2 may have a common mechanism of nuclear import (Gelvin 2003).

AGROBACTERIUM-MEDIATED TRANSFORMATION OF PLANT VEGETATIVE CELLS

Most methods of *Agrobacterium*-mediated transformation are based on the coincubation of plant vegetative organs and tissues (seeds, leaves, roots, stems, or meristems) with bacterial-cell suspensions (Feldmann and Marks 1987; Feldmann 1992; Katavic *et al*. 1994; Bent and Clough 1998; Clough and Bent 1998; de la Riva *et al*. 1998; Chung *et al*. 2000; Kojima *et al*. 2006).

However, each of them has certain disadvantages, which led to research into the development of novel alternative systems such as infiltration, electroporation of cells and tissues, electrophoresis of embryos, microinjection, pollen-tube pathway, silicon carbide- and liposome-mediated, floral dip transformation methods (Rakoczy-Trojanowska 2002; Bent 2006). The low efficiency of transformation is

considered to be the main reason for the limited popularity of the alternative transformation methods, other than infiltration and silicon carbide-mediated transformation, which seem the most promising ones (Rakoczy-Trojanowska 2002).

Agrobacterium-mediated transformation of germinating seeds of *Arabidopsis thaliana* as a non-tissue culturing approach was first demonstrated about 20 years ago (Feldmann and Marks 1987). It is currently used widely as a method of *Agrobacterium* T-DNA-mediated insertion mutagenesis to study the structural and functional organization of the plant genome. Production of insertion mutants by *in planta* transformation seems especially promising for monocotyledonous plants, since there are the difficulties of plant regeneration from suspension and callus cultures.

Kojima *et al*. (2006) developed a general *in planta* transformation method in which *A*. *tumefaciens* is inoculated onto the meristems of various parts of soaked seeds, seedlings, or young plants (depending on the plant), and the inoculated plants are grown to maturation in pots under nonsterile conditions. The transformation efficiency of all buckwheat, mulberry, kenaf, and rice plants transformed by this method was extremely high, compared with those of the general *in vitro* transformation methods reported previously (Kojima *et al*. 2006) The authors assumed that *in planta* transformation probably mimicked *A*. *tumefaciens* infection of plants in nature. This might account for the high transformation efficiency of this method (Kojima *et al*. 2006).

In 1993, Bechtold *et al*. (1993) reported on an original method for transformation of *Arabidopsis* by *Agrobacterium* vacuum infiltration. The primary reasons for the popularity of this method have been its simplicity and reliability. With vacuum infiltration and other *in planta* transformation methods, most transformed progeny are genetically uniform (nonchimeric), and the somaclonal variation associated with tissue culture and regeneration is minimized (Tague and Mantis 2006). The elimination of tissue culture and regeneration greatly reduces hands-on time, and success can be achieved by nonexperts (Bechtold *et al*. 1993; Bent and Clough 1998).

A method of transgenic-plant generation through the coincubation of immature plant embryos with *Agrobacterium* cells followed by multiple callus passages was suggested (Zhao *et al*. 2000). The level of stable plant transformation achieved by this approach, calculated relative to the inoculated calli, was 10%. The disadvantages of the method are the high labor expenditure and the long duration of the process of transgenic-plant production. Furthermore, the use of *in vitro* cultivation systems can lead to the formation of somaclonal plant variants.

It seems that the tested plant-generative-cell-transformation approach is easy and cheap, compared to the others, which require the use of expensive cultural media for the production of callus cultures and for the subsequent plant regeneration. The proposed approach makes it possible to generate transgenic plants without *in vitro* cultivation. Consequently, there is no danger of appearance of somaclonal variation. Furthermore, the application of this approach is not restricted to genotypes capable of forming well-growing embryogenic callus. The method of generative-cell transformation seems most promising for monocotyledonous plants, since there are the difficulties of plant regeneration from suspension and callus cultures.

Factors influencing the effectiveness of agrobacterial transformation

Temperature

Temperature is a key factor in agrobacterial-mediated plant transformation (Tempe *et al*. 1977). To date, several transformation stages sensitive to high temperature have been described for Vir proteins (Fullner and Nester 1996; Chumakov *et al*. 2002). The temperature range of 19 to 22°C is optimal for the expression of *vir* genes in *Agrobacterium* (Fullner and Nester 1996). In particular, 28°C is critical for the excretion and assemblage of *vir*-dependent extracellular agrobacterial T-pili, essential for successful T-DNA transfer (Fullner *et al*. 1998). Transfer of T-DNA into tobacco plantlets under laboratory conditions was completely suppressed at 31°C (Chumakov *et al*. 2002). Temperature (22-25°C) during silk treatment with an agrobacterial suspension was less favorable for *Agrobacterium*-mediated transformation compared to 18-20°C (Chumakov *et al*. 2006). These findings seem to conflict with the data on the temperature effects on T-DNA transfer machinery inside the agrobacterial cells. Since activation of the *vir* genes in *Agrobacterium* and subsequent incubation of the bacterial cells under laboratory conditions were performed at 27°C and were followed by coincubation with the plant at 18-25°C, a possible explanation could be the low temperature-sensitivity of the transformation stages taking place in the plant-host cells. This suggestion is supported by the confirmed transfer and integration of T-DNA into the animal cell nuclei after coincubation of *Agrobacterium* and HeLa cells at 37°C (Kunik *et al*. 2001).

Cooling for a short period (for 4 h under light conditions) held back the transformation by *A*. *tumefaciens* with activated *vir* genes of undamaged tobacco seedlings, since low-temperature pretreatment probably affects the state of the cytoskeleton (Chumakov *et al*. 2002). In addition to the temperature factor, the frequency of T-DNA transfer and integration can be influenced by the conditions for virulence-gene induction (AS concentration and induction time).

Effects of light and cytoskeleton

Illumination (light) as a factor limiting *Agrobacterium*-mediated transformation of uninjured tobacco plantlets was first demonstrated by Chumakov *et al*. (2002) and was confirmed by Zambre *et al*. (2003). The number of transformed (GUS-positive) tobacco plantlets dropped two-fold after incubation in darkness for 1 h; 90% of the leaf stomata were closed. The authors concluded that transformation is related to the state of plant stomata and plasmodesmata and to light- and temperature-dependent cytoskeleton effects (Chumakov *et al*. 2002). A positive effect of light (in a 16 h light/8 h dark photoperiod) during cocultivation of *A*. *tumefaciens* with callus from two genotypes of the crop plant *Phaseolus acutifolius* (tepary bean) and also during cocultivation of root segments was observed by Zambre *et al*. (2003). The observed positive effect of light has obvious implications for the development and improvement of transient and stable transformation protocols, specifically those involving cocultivated in the dark (Zambre *et al*. 2003).

However, dark incubation can also change the state of the plant cytoskeleton (Gamalei 1996). When two-week-old tobacco seedlings were preincubated with 0.1 mM colchicine (which disassembles actin microfibrils and affects the cytoskeleton in tobacco protoplasts; Smith and Raikhel 1998) 30 min before the incubation with agrobacteria, 50% of the stomata were closed, and the number of transformed cells dropped two- to three-fold (Chumakov *et al*. 2002).

Factors that produce significant differences (0.3-3.3%) in T-DNA delivery to immature embryos from a range of plant (wheat) varieties and in regeneration include the embryo size, the duration of preculturing, inoculation and cocultivation, and the presence of AS and a surfactant in the media (Wu *et al*. 2003).

Agrobacterium-mediated "floral dip" method for plant-gametophyte-cell transformation

For the transformation of germ cells, a simple method was suggested that consists in dipping male and female inflorescences of *Arabidopsis* into a suspension of *Agrobacterium* cells with activated virulence genes (Clough and Bent 1998; Bent 2006). Earlier versions of this method and related methods have been known as "*Agrobacterium* vacuum infiltra-

tion" or *"in planta* transformation" (Feldmann 1992; Bechtold *et al*. 1993; Chang *et al*. 1994; Katavic *et al*. 1994). With respect to the present method, these names are either inaccurate or ambiguous in that they have been used previously to describe substantially different methods. To provide a distinct name that is descriptive of the process, Clough and Bent (1998) proposed the term 'floral dip' for methods in which plants are transformed by direct application of *Agrobacterium* to floral tissues, without the use of vacuum or other pressurizing devices. Veit *et al*. (2006) effectively used the floral dip method to introduce phosphinotricin-resistant genes into 6-day-old *Chenopodium rubrum* seedlings.

The level of transformation in the case of Clough and Bent (1998) was 4% in the first generation. The efficiency of the floral dip approach was similar to that of the *Agrobacterium* suspension spray on the flowers of *Arabidopsis* (Chung *et al*. 2000). The floral-spray method opens up the possibility of *in planta* transformation of species that are too large to be used in dipping or vacuum infiltration.

The mechanism bringing T-DNA into germ cells is unknown. A possible way of T-DNA delivery into the nuclei of maternal embryo-sac cells of maize might be the delivery of *Agrobacterium* into the egg cell with a growing pollen tube (Chumakov *et al*. 2006).

Plant-growth stage

Of the many variables present in the germ-line transformation protocol, the developmental stage of *A. thaliana* at the time of inoculation with *Agrobacterium* is one of the most important. The optimal growth stage for *Agrobacterium*-mediated transformation of *A. thaliana* by the floral dip procedure is when plants contain numerous unopened floral buds (Clough and Bent 1998).

Inoculation-medium effects

Clough and Bent (1998) found that the absence of Murashige and Skoog medium salts and pH adjustment had no effect on *Agrobacterium*-mediated transformation rates, but sucrose and surfactant in the inoculation medium affected transformation by the vacuum infiltration method.

When Liu *et al*. (1992) attempted to preinduce the *vir* genes by growing *Agrobacterium* for 20 h in a standard *vir*-inducing liquid medium instead of a rich liquid medium, the rate of transformation did not change significantly. Growth on a solid *vir*-inducing CIB medium (Fullner *et al*. 1994) at 19°C led to a four-fold decrease in the transformation rate.

When *Agrobacterium* was grown to a stationary phase and then resuspended at various inoculum concentrations ranging from OD_{600} 0.15 to 1.75, there was little difference in the transformation rate. It also was found that cells grown to very late stationary phase (prepared from cultures grown for 84 h) and diluted to OD_{600} 0.8 transformed just as efficiently as did the younger cultures (I. Volokhina, pers. comm.).

The efficiency of the floral dip method can be increased by nearly three-fold by using 0.05% (v/v) Silwet L-77 surfactants. However, in general, the efficiency of transgenic-plant generation remains low. For instance, in the first generation of radish it constituted 0.5 to 1.4% (Curtis and Nam 2001). With this approach, it is possible to obtain up to 2.7% of Southern-positive transformants in wheat (Pukhalskiy *et al*. 1996) and 6.7% PCR-positive transformants in corn (Chumakov *et al*. 2006).

Targets for *Agrobacterium*-mediated plant transformation of germ-line cells

When *Arabidopsis* flowers are dipped into an *Agrobacterium* suspension, the ova and tissues of the female gametophyte become a target for T-DNA because treatment of the male plants (anthers) does not produce transgenic plants (Desfeux *et al*. 2000). A critical step is the flower treatment time. A similar approach to foreign-gene transfer through the flowering spikes by using agrobacterial cells with acetosyringone-activated *vir* genes has been undertaken earlier in wheat (Pukhalskiy *et al*. 1996), but the frequencies obtained from those experiments were defined as 'low'.

Integration of T-DNA carrying the *npt*II gene into the maize genome as a result of treatment of pistil filaments with *Agrobacterium* cells containing activated *vir* genes *ex planta* was demonstrated by Chumakov *et al*. (2006). A cell suspension of *A. tumefaciens* carrying activated *vir* genes was applied onto the previously isolated pistil filaments, which were afterwards pollinated with the pollen of the same cultivar. With this approach, the output of transgenic plants can be as high as 60% of the number of kanamycin-resistant seedlings grown from the transformed seeds, which constitutes up to 7% of the total number of seeds.

The high frequency of genetic transformation in these investigations may have resulted from a successful combination of different factors. First, one should note that the need for a time interval between pollination and inoculation of an agrobacterial cell suspension is very important. In experiments in which inoculation of an agrobacterial suspension immediately followed pollination, the seed in an ear was completely absent, perhaps as a result of inhibition of the pollen-tube growth factors (Chumakov *et al*., unpublished data). At the same time, application of the agrobacterial suspension 4–7 hours after pollination, i.e., when the pollen tube reached the female gametophyte, allowed Mól *et al*. (2004) to obtain kernels with transgenic embryos. *Agrobacterium* can either independently, with the use of the virulence proteins VirE2 and VirD2, secure T-DNA transfer though the membrane and cytoplasm into the nucleus, after the contact with the female gametophyte cells, or it can be absorbed during the entrance of the sperm cell nuclei into the egg cells.

Analysis of the first-generation *Arabidopsis thaliana* plants showed that T-DNA inserts were present in one of the alleles. The insert homozygous plants were very rare or absent. This finding suggests that the integration takes place in the developing embryo. Moreover, the embryo-sac cells are transformed independently (Bechtold *et al*. 2003).

Taking into account this fact, we speculate that genetic transformation in this experimental system proceeds by the pollen-tube pathway, with the agrobacterial cells either transforming the pollen tube and then the zygote or hitting directly the zygote following the trace of the pollen tube (Chumakov *et al*. 2006). It is known that the pollen tube moves towards the micropyle (the germ pore). This movement is guided by an attractor protein produced by the micropyle cells (Marton *et al*. 2005).

The productive transformation events occur on female floral structures and do not occur during the early stages of anther or pollen/microspore development prior to pollen release (Desfeux *et al*. 2000). It is possible that multiple developmental stages serve as productive targets, ranging from the ovule primordia that will give rise to the megasporophyte, through any stage of megagametophyte development, to the recently fertilized embryo.

CONCLUDING REMARKS

Most of the methods of *Agrobacterium*-mediated transformation are based on coincubation of plant vegetative organs and tissues (leaves, roots, or stems) and the bacterial-cell suspension. The adult plants are then regenerated from the cultivated cells or tissues. This approach can result in the production of chimeric transformants or has certain disadvantages, which led to research into the development of novel alternative systems such as infiltration, electroporation of cells and tissues, electrophoresis of embryos, microinjection, and the pollen-tube-pathway and floral-dip-transformation methods. With "floral dip" and other *in planta* transformation methods, most transformed progeny are genetically uniform (nonchimeric), and the somaclonal varia-

tion associated with tissue culture and regeneration is minimized. The elimination of tissue culture and regeneration greatly reduces hands-on time, and success can be achieved even by nonexperts.

Production of insertion mutants by "floral dip" and *in planta* transformation seems especially promising for monocotyledonous plants, since there are the difficulties of plant regeneration from suspension and callus cultures. But the efficiency of transformation by these transformation methods is still low, and it is necessary to understand unknown aspects of the mechanism of T-DNA transfer if we are to improve agrobacterial transformation technologies.

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