establish the health and environmental risks from engineered nano-scale particles. Although research on the adverse effects of NPs on human health is progressing rapidly, environmental fate of NPs is still in its infancy. Before unknowingly dumping a huge amount of dangerous nanomaterials into the environment, we need to investigate the solubility and degradability of engineered NPs in soils and waters, to establish baseline information on their safety, toxicity and adaptation of soil and aquatic life. Development of novel NPs must be followed by the assessment of their potential risks on life and environment, and possible remedial measures.

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A modified freeze–thaw method for efficient transformation of *Agrobacterium tumefaciens*

There is a great potential for genetic manipulation of crop and medicinal plants to enhance productivity through increasing pest and microbial disease resistance and environmental stress tolerance, and also for studying gene function and regulation of physiological and developmental processes[1]. Transgenic plants that transmit the introduced trait to progeny generations are produced using various DNA delivery methods such as particle acceleration[2], electroporation[3] and polyethylene glycol permeabilization of protoplasts[4]. However, most commonly used method for obtaining transgenic plants is by the *Agrobacterium tumefaciens*-mediated transformation[5,6]. *Agrobacterium* can transfer DNA to a remarkably broad group of organisms – numerous dicot and monocot angiosperm species[5], gymnosperms[6] and fungi, including yeast[7], ascomycetes[8] and basidiomycetes[9]. Recently, *Agrobacterium* was reported to transfer DNA to human cells[10].

The general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First is the cloning of the desired gene into binary vector and transforming the same into *Escherichia coli*. Secondly, the binary vector construct is mobilized from *E. coli* to *Agrobacterium* by triparental mating[11], or direct introduction of the genetically engineered binary vector construct into *Agrobacterium* by electroporation[12] or freeze and thaw method[13]. Triparental mating requires at least five to seven days in order to determine the successful mobilization into *Agrobacterium* and is confined to strains harbouring plasmids that carry the *mob* gene[11]. Electroporation is faster and more efficient than triparental mating, but requires special equipment. Till date, the transformation frequency reported by freeze–thaw method in *Agrobacterium* has remained low[13–15]. Here we describe a simple and reliable protocol for competent cell preparation and efficient transformation by freeze–thaw method in *A. tumefaciens* LBA4404.

A. tumefaciens strain LBA4404 (pAL 4404) was procured from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. The binary vector used for transformation was the pGreen-CaCPK2 (calcium-dependent protein kinase isoform 2 cDNA isolated from *Cicer arietinum* L.) construct, with the neomycin phosphotransferase (*npt*II) gene as a selectable marker (confers to kanamycin resistance). *A. tumefaciens* strain LBA4404 was streaked out on a LB[16] plate containing 1 mg/l rifampicin and grown at 28°C overnight. A single colony was used to inoculate 3 ml of LB medium containing 1 mg/l rifampicin and grown overnight at 28°C, shaking at 160 rpm. A 50 ml of LB medium with 1 mg/l rifampicin was inoculated with 0.5 ml (1/100th volume) of the overnight culture and grown in an incubator-shaker at 28°C at 160 rpm to obtain cell densities of 0.3, 0.4, 0.5 and 0.6 at OD_{600}. The cultures were chilled on ice for 15 min and the cells were then harvested by centrifugation at 3000 rpm for 5 min at 4°C, resuspended in 10 ml of sterile ice-cold 50 and 100 mM $MgCl_2$ solutions, and incubated on ice for 1 h. After another centrifugation step as above, the resulting pellet was resuspended in

sterile ice-cold 2 ml of 20 mM $CaCl_2$, and incubated on ice for 6 h to yield the competent cell suspension. Glycerol was added to a final concentration of 20% and aliquots of 100 µl were frozen in liquid N_2 and stored at –70°C for later use.

Glycerol stocks of competent cells (100 µl) were thawed on ice for 5 min and 1 µl of pGreen-CaCPK2 construct (1 µg/µl) was added to it, mixed by gently tapping and frozen in liquid N_2 for 10 min and then thawed at 37°C for 5 min. Then 10 µl of this mixture was inoculated into 1 ml of pre-warmed LB medium without antibiotics and incubated for 1 h at 28°C in a rotory shaker at 160 rpm. Next 50 µl of the suspension was spread onto LB plates containing 1 mg/l rifampicin and 50 mg/l kanamycin. After incubation at 28°C for 24 h, colonies are counted. Transformation efficiency is defined as the number of colony forming units (CFU) divided by 1 µg of plasmid DNA[17].

$$\text{Transformant CFU} = \frac{[\text{Number of bacterial colonies} \times \text{dilution ratio} \times \text{original transformation volume}]}{\text{Plated volume}}$$

$$\text{Transformation efficiency} = \frac{\text{Transformant CFU}}{\text{Plasmid DNA (µg)}}.$$

A. tumefaciens cells grown in LB medium supplemented with rifampicin at OD_{600} 0.3, 0.4, 0.5 and 0.6 were taken for the competent cell preparation by divalent cations, $MgCl_2$ and $CaCl_2$ treatment method. $MgCl_2$ at 50 and 100 mM concentrations with 20 mM $CaCl_2$ was tested for competent cell preparation and transformation efficiency. The optimum concentration of $MgCl_2$ needed for efficient transformation of *A. tumefaciens* was found to be 100 mM. Transformation efficiency was low in competent cells prepared with 50 mM $MgCl_2$ and 20 mM $CaCl_2$ concentrations (data not shown). The role of these divalent cations in transformation of *A. tumefaciens* is not known. However, it is well established in *Diplococcus pneumoniae*[18,19], wherein the presence of both magnesium and calcium ions improves the levels of genetic transformation by 30% to twofold over that obtained in the presence of calcium only.

A. tumefaciens at different growth rates for transformation efficiency with pGreen-CaCPK2 construct was investigated. It was found that OD_{600}-0.3 was the most potent with maximum transformation efficiency of 3.6×10^5 transformants/µg DNA, whereas it gradually declined from OD_{600}-0.3 to 0.6 cultures (Table 1). It is important that the bacterial cells must be in their early logarithmic period for efficient transformation, as evident from earlier reports[12,14,17]. *E. coli* DH5α and *E. coli* BL 21 strains-proved to be highly efficient at early logarithmic phase for divalent cations-treated competent cell preparation and freeze–thaw transformation with recombinant pTZ57R/T and pRSET vectors in our laboratory[20].

Transformation frequency of the binary vector pGreen-CaCPK2 construct under optimum conditions (greater than 10^5 transformants/µg DNA) was significantly higher than those previously reported for *A. tumefaciens*[13]. The main factors that contribute greatly to the efficiency of transformation were the use of early logarithmic phase cells (OD_{600}-0.3) and inclusion of 100 mM $MgCl_2$ in the preparation of competent cells.

An interesting aspect of transformation is that the efficiency depends on a particular combination of bacterial strains and plasmid-selection markers[21]. The different binary vectors of almost same molecular size with different selectable markers were used for transformation of *Mesorhizobium loti* by freeze–thaw method. It was found that pPZP211 with spectinomycin resistance gene was highly efficient with 1.6×10^5 transformants/µg DNA compared to pART27 and pSoup with kanamycin and tetracycline selectable markers respectively[22]. Therefore, the transformation efficiency obtained in our experiment can be compared to that of *A. tumefaciens* LBA4404 transformed with pDG12Sa binary plasmid vector with kanamycin resistance gene via electroporation, wherein $1–1.5 \times 10^6$ transformants/µg DNA was achieved[23].

A. tumefaciens transformants were tested for the presence of pGreen-CaCPK2 construct by PCR analysis with gene-specific primers for *CaCPK2* and *npt*II genes. The plasmid DNA from *A. tumefaciens* was isolated using Sigma plasmid isolation kit according to the manufacturers' protocol. The primers for *CaCPK2* gene were 5′-ATGGGTAATT GTTGCGCTACCCCTC-3′ (*CaCPK2*-F), and 5′-CGACCTAGCTCGTATCG-3′ (*CaCPK2*-R) and for *npt*II gene were 5′-GCACAACAGACAATC-3′ (*npt*II-F) and 5′-CCGCCAAGCTCTTCA-3′ (*npt*II-R). All the reactions were carried out in a 50 µl volume containing 100 ng of plasmid DNA, 0.1 mM dNTP mix, 0.2 µM of each primer, and 1.25 U/rxn *Taq* DNA polymerase (Bangalore Genei, India) with 1× *Taq* reaction buffer containing 1.5 mM $MgCl_2$. The PCR was performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc., USA) programmed as follows: Initial denaturation of 1 min at 94°C; followed by 30 cycles of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C, and a final synthesis at 72°C for 10 min for *CaCPK2* gene. For the *npt*II gene the initial denaturation of 1 min at 94°C was followed by 30 cycles of 15 s at 94°C, 15 s at 56°C, 30 s at 72°C; and a final synthesis at 72°C for 5 min. PCR products were resolved on 1% (w/v) agarose gel in 1× TAE buffer stained with ethidium bromide and digitized using UVIPRO (Uvitec, Cambridge, UK) digital imaging system.

PCR analysis showed the presence of the 1.6 kb amplicon (Figure 1, lane 4) and 600 bp amplicon (Figure 1, lane 3) corresponding to the *CaCPK2* and *npt*II genes respectively. These data were also confirmed by Southern blot analysis.

The plasmids (2–5 µg) from untransformed and transformed *A. tumefaciens* were digested with *Pst*I alone and Southern

Table 1. Transformation efficiency of *Agrabacterium tumefaciens* with pGreen-CaCPK2 construct determined using competent cells isolated from different cell densities of the culture

A. tumefaciens competent cells at OD_{600}[a]	Transformation efficiency (transformants/µg DNA)[b]
0.3	3.6×10^5
0.4	2.4×10^4
0.5	1.8×10^4
0.6	1.2×10^3

[a]OD_{600}, Optical density at 600 nm. [b]Colony forming units/µg of pGreen-CaCPK2 DNA.

blot analysis was performed using a standard protocol[16]. The 1.6 kb *CaCPK2* cDNA was radiolabelled with [α-^{32}P] dCTP by random primer labelling and used as the probe. The *Pst*I digested pGreen-CaCPK2 construct isolated from transformed *A. tumefaciens* resulted in the release of 35S CaMV promoter with *CaCPK2* gene [2.3 kb] and partially digested pGreen backbone with *CaCPK2* gene (6.9 kb) (Figure 2 *a*, lane 4). Southern blot hybridization with a [α-^{32}P] dCTP-labelled *CaCPK2* gene probe showed double bands (Figure 2 *b*, lane 4), indicating the presence of pGreen-CaCPK2 construct.

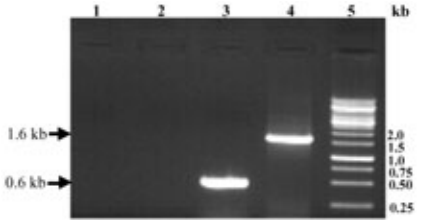

Figure 1. PCR analysis of transformed *Agrabacterium tumefaciens*. Plasmid DNAs isolated from transformed and untransformed *A. tumefaciens* were provided as the templates in PCR reactions using *CaCPK2* and *npt*II gene-specific primers. Lane 1, pAL 4404 (untransformed *A. tumefaciens*) with *CaCPK2* primers; lane 2, pGreen 0029 (*Escherichia coli* DH5α) with *CaCPK2* primers; lane 3, pGreen-CaCPK2 construct (transformed *A. tumefaciens*) with *npt*II primers; lane 4, pGreen-CaCPK2 construct (transformed *A. tumefaciens*) with *CaCPK2* primers, and lane 5, 1 kb DNA marker. Arrows indicate the expected amplicons of 1.6 kb *CaCPK2* and 600 bp *npt*II genes.

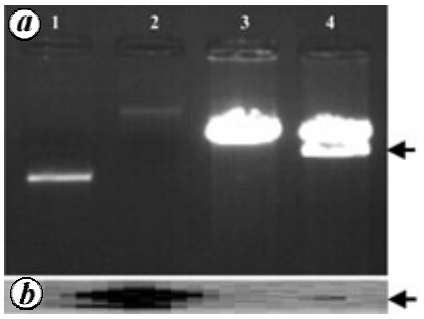

Figure 2. Agarose gel electrophoresis and Southern blot analysis of *A. tumefaciens* transformed with pGreen-CaCPK2 construct. ***a***, Plasmid DNA was isolated from untransformed and transformed *A. tumefaciens*, digested with *Pst*I, resolved on 1% agarose gel, and transferred to nylon membrane. ***b***, DNA from agarose gel was blotted onto a nylon membrane and probed with (α-^{32}P) dCTP-labelled *CaCPK2* gene probe. Lane 1, Positive control *CaCPK2* fragment; lane 2, pAL 4404 (untransformed *A. tumefaciens*); lane 3, pGreen 0029 (*E. coli* DH5α), and lane 4 pGreen-CaCPK2 construct (transformed *A. tumefaciens*). Arrows indicate a 2.3 kb fragment of 35S CaMV promoter with *CaCPK2* gene.

Agricultural biotechnology is dependent on using *Agrobacterium* to create genetically modified crops in order to obtain the desired agronomic traits like pest and disease resistance, and herbicide and stress tolerance. These transgenic crops have the potential to produce widespread social and economical benefits, particularly for developing countries. Usage of *Agrobacterium* is not only confined to plants but it also succeeded in transferring genes to human cells[10], suggesting the exciting possibility of human and animal gene therapy. In these pursuits, a simple, rapid and reliable protocol as presented here can be effectively used to transform *A. tumefaciens* with binary plasmid vector construct by freeze–thaw method.

In summary, an improved freeze–thaw method offering efficient transformation of *A. tumefaciens* LBA4404 with a binary vector plasmid pGreen-CaCPK2 construct has been described. Bacterial cultures grown in LB medium at OD_{600}-0.3, 0.4, 0.5, and 0.6 were treated with 100 mM $MgCl_2$ and 20 mM $CaCl_2$ for competent cell preparation and transformed with pGreen-CaCPK2 construct by freezing in liquid N_2 followed by thawing at 37°C. The procedure yields maximum transformation efficiency of 3.6×10^5 transformants/µg of DNA in OD_{600}-0.3 cultures. Transformation was further confirmed by PCR and Southern analysis. This improved freeze–thaw method is a simpler procedure to transform *Agrobacterium* directly with a binary vector plasmid.

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