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Shorter T-DNA or additional virulence genes improve *Agrobactrium*-mediated transformation

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Abstract The effect of additional virulence (*vir*) genes and size of plasmid T-DNA in *Agrobacterium tumefaciens* was investigated for their impact on transformation efficiency. Transformation efficiency in tobacco, cotton, and rice was increased when the T-DNA was 4.3 kb compared to 8.4 kb in size. However, when additional *virG*, *virG*N54D, *virE*, or *virE*/*virG* plasmids were included with the 8.4-kb T-DNA, transformation frequencies in all cases were increased over that of the shorter T-DNA without additional *vir* plasmids. The use of *virE*, *virG* or *virG*N54D copies enhanced transformation efficiency; however, the most significant increase of transformation efficiency in all three plant species was observed when the *virE*/*virG* plasmid was used for infection. The *virE*/*virG* plasmid dramatically enhanced the efficiency of *Agrobacterium*-mediated gene transfer; moreover, this plasmid appears to have broad efficiency since it was consistently effective on two different dicotyledon species as well as a monocotyledon species.

Key words *Agrobacterium* · Rice · Cotton · Tobacco · Virulence genes

Introduction

Agrobacterium-mediated transformation often results in single- or low-copy integration of full-length T-strand DNA, and such simple insertion events tend to result in stable expression (Klee et al. 1987). Transfer of T-DNA to shoot apices using *A. tumefaciens* is an effective method since most shoot apices can reform intact, genotypically stable plants. Strong transient expression of β-glucuronidase (GUS) was observed in apical tissues of shoots cocultivated with *A. tumefaciens* (Shen et al. 1993; Hiei et al. 1994). This explant can successfully be used in *Agrobacterium*-mediated monocotyledon and dicotyledon transformation (Hussey et al. 1989; Ulian et al. 1989; Schrammeijer et al. 1990; Gould et al. 1991; Bidney et al. 1992; May et al. 1995; Park et al. 1996; Zimmerman and Scorza 1996; Zapata et al. 1999). However, a critical problem limiting the use of the shoot apex is that cells differentiating into the germline and eventually giving rise to reproductive tissues are several cell layers from the surface. The production of transgenic plants from these tissues might be difficult unless the transformation efficiency is extremely high (Lowe et al. 1995; Hiei et al. 1997).

Factors such as the type of *Agrobacterium* strain, the effect of temperature, the presence of *vir* gene-inducing substrates, and the effect of additional *vir* gene copies have been investigated to enhance transformation efficiency in this laboratory as well as in several other laboratories (Liu et al. 1992; Hansen et al. 1994; Hiei et al. 1994; Hamilton et al. 1996; Dillen et al. 1997). One of the most critical factors in our laboratory appears to be the effect of additional *vir* gene copies. Hamilton et al. (1996) suggested that additional copies of *virG* and *virE* genes enhanced the transfer of high-molecular-weight T-DNA (at least 150 kb) into tobacco genomic DNA. In addition, Liu et al. (1992) and Hansen et al. (1994) demonstrated that additional copies of *virG* or *virG*N54D (caused constitutive expression of other *vir* genes independent of *virA*) in *A. tumefaciens* enhanced the transient transformation frequency using GUS expression assays.

In this paper, the effect of additional copies of *virG*, *virG*N54D, *virE*, or *virE*/*virG* and two sizes of T-DNA in *A. tumefaciens* LBA4404 were evaluated using the tobacco leaf disk and the cotton and rice shoot apex transformation systems. Phenotype expression of herbicide (*pat* gene) and insect (*bt* gene) resistance as well as molecular analysis of some plants were examined. Additionally, the effect of the *virE*/*virG* plasmid was analyzed using green fluorescent protein (GFP) expression assays in cotton shoot apices.

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Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38), cotton (*Gossypium hirsutum* L. cv. Sphinx), and rice (*Oryza sativa* L. cv. Jefferson) seeds were used in this study. All culture conditions for germination, preparation of tobacco explants, and shoot apex isolation were established according to Park et al. (1998a, b).

Bacterial strain and plasmids

An octopine strain of *A. tumefaciens*, LBA4404, was used. Three plasmid constructs, pAGM 277 containing the in proof copy *ubi/pat/orf25* expression vector, pAGM281 containing the *ubi/pat/orf25\bt\(4Ocs) Δmas* expression vector, and pBIN m-gfp5-ER containing the *nos/nptII/nos-ter/35S/m-gfp5-ER/nos-ter* expression vector were used. The helper plasmids, pAD1289 carrying the mutant *virG*N54D (from the octopine Ti plasmid pTiA6), pCH30 carrying *virG* (from the L, L-succinamopine Ti plasmid pTiBo542), pCH32 carrying *virG* (pTiBo542)/*virE1virE2* (pTiA6), and pCH42 carrying *virE1virE2* (pTiA6), were mobilized into *A. tumefaciens* LBA4404 containing pAGM281 by the freeze-thaw method (Holsters et al. 1978). Plasmid pCH32 was also mobilized into *A. tumefaciens* LBA4404 containing pBIN m-gfp5-ER.

Plant transformation

In order to establish the most effective selection levels, we doubled the lowest level of selective agent that completely inhibited control growth. This double concentration of selective agent minimized the escapes that can result in cross-protection and/or transient expression. Therefore, levels of 4 mg l^{-1} glufosinate-ammonium (ppt) for tobacco, 2.5 mg l^{-1} ppt for cotton and 4 mg l^{-1} ppt for rice were chosen for selection.

Tobacco transformation was performed as described by Horsch et al. (1985). Leaf disks of equal size were initially cultured on the same MS inorganic salts medium (Murashige and Skoog 1962) on which the germinated seedlings were grown plus 1 mg l^{-1} N^6-benzyladenine (BA), 0.1 mg l^{-1} α-naphthalene acetic acid (NAA), 3% (w/v) sucrose, and 0.8% (w/v) TC agar at pH 5.7 for 1 day. The leaf disks were subsequently inoculated with *A. tumefaciens* (OD_{600} of 1.2) cultured on YEP medium (10 mg l^{-1} yeast extract, 10 mg l^{-1} trypton, 5 mg l^{-1} NaCl) containing the appropriate antibiotics for 2 min and then cocultivated on a complete MS medium for 72 h. They were transferred to selection medium containing 250 mg l^{-1} Clavamox (amoxicillin trihydrate, SmithKline Beecham, West Chester, Pa.) and 4 mg l^{-1} ppt. The possible enhancing effect of additional *vir* genes on plant transformation was examined by assaying the transformation efficiency using tobacco. Additional copies of *virG*, *virG*N54D, *virE*, and *virE*/*virG*, respectively, were introduced into *A. tumefaciens* LBA4404 containing pAGM281. After leaf disk cocultivation with *A. tumefaciens* containing different *vir* genes, plants were regenerated from leaf disks on 4 mg l^{-1} ppt. Transformation efficiency was evaluated on the basis of the number of leaf disks with ppt-resistant regenerated plants. Leaf disks showing pale, vitrified shoot development were not counted. To compare transformation efficiency we used leaf disks of equal size and equal concentrations of *A. tumefaciens*. The number of leaf disks with ppt-resistant regenerated plants was scored 6 weeks after cocultivation.

Cotton transformation was performed as described by Zapata et al. (1999). Shoot apices were inoculated in an *A. tumefaciens* suspension (OD_{600} of 0.8–0.9) for 5 min and then cocultivated on filter paper saturated with liquid MS inorganic salts supplemented with 100 mg l^{-1} of myo-inositol, 0.5 mg l^{-1} thiamine·HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine·HCl, 3% (w/v) sucrose, and 0.15% (w/v) Gelrite at pH 5.7 for 72 h. After cocultivation, the bacteria were removed by several washes in Clavamox solution (250 mg l^{-1}) and sterile distilled water rinses. Shoots were then established on the MS medium described above with the addition of 250 mg l^{-1} Clavamox for 5 days. They were then transferred to selection medium containing 250 mg l^{-1} Clavamox and 2.5 mg l^{-1} ppt.

Rice transformation was performed as described by Park et al. (1996). Shoot apices were initially cultured on MS inorganic salts, 0.5 mg l^{-1} kinetin, 0.05 mg l^{-1} NAA, 100 mg l^{-1} myo-inositol, 0.1 mg l^{-1} of thiamine·HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine, 3% (w/v) sucrose, and 0.8% (w/v) TC agar at pH 5.7 for 1 day. They were then inoculated with *A. tumefaciens* (OD_{600} of 1.2) cultured on YEP medium containing the appropriate antibiotics and cocultivated on the MS medium described above for 72 h. Finally they were then transferred to selection medium containing 250 mg l^{-1} Clavamox and 4 mg l^{-1} ppt.

Acetosyringone (100 μ*M*) was used in all experiments and was added 2 h before cocultivation. Cultures were maintained at 24–25°C for 16 h under 60–80 μE m^{-2} s^{-1} light.

DNA isolation and slot-blot analysis

Cotton genomic DNA was extracted from leaf tissue according to Paterson et al. (1993). Isolated DNA was blotted onto a nylon membrane (Zeta-probe GT membrane, Bio-Rad, Hercules, Calif.) according to the manufacturer's directions. DNA was fixed to the membrane by baking at 80°C for 30 min and hybridized to DNA fragments labeled with [^{32}P]-dCTP using a random primer DNA labeling system (BRL, Gibco BRL, Gaitherburg, Md.). The probe for PAT was made from a *Sal*I restriction fragment of the pAGM102 plasmid (558 bp fragment). The hybridization and washing conditions have been described previously (Park et al. 1998b). Filters were exposed to X-ray film at –70°C.

Herbicide resistance test

Out of 79 rice ppt-resistant regenerated plants 40 were randomly chosen for the herbicide resistance test. Isolated shoot apices were cultured on callus induction medium after cocultivation with *A. tumefaciens* containing pAGM281. After 5 weeks on callus induction medium (the same rice MS medium described above with 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid instead of 0.5 mg l^{-1} kinetin, and 0.05 mg l^{-1} NAA) calli derived from shoot apices were then each cut into approximately 15–20 small pieces and transferred to selection medium. During subsequent rounds of selection, proliferating and apparently resistant calli were cut up further, whereas growth-inhibited and browning calli were removed.

Insect feeding bioassay

Leaf disks from ppt-resistant regenerated plants of tobacco and cotton were cut from recently expanded leaves (2 disks per each leaf cut into 1.2-cm-diameter disks) and placed on a moistened filter paper in a 60×15-mm petri dish. Five tobacco budworm (*Heliothis virescens*) larvae were allowed to feed for 4 days. This test was maintained at 25°–26°C for 16 h under 60–80 μE m^{-2} s^{-1} light.

Detection of GFP by the epi-fluorescent microscope

Cotton shoot apices were examined using an epi-fluorescent microscope (Axioskop I, Zeiss) with a UV light source and a Chroma Technology filter set providing a HQ470/40 exciter, a HQ525/50 emitter, and a Q495LP beamsplitter. No significant autofluoresence was detected in any of the negative controls using this filter combination. Photographs were taken using KODAK Elite II film (400 ASA). Magnification at 100× or 25× and short exposure times were used.

Results

R_o plant analysis

After explants had been cocultivated with *A. tumefaciens* containing pAGM281, 20 out of 32 tobacco and 40 out of 65 cotton ppt-resistant regenerated plants were randomly selected for the insect feeding bioassay. Tobacco budworm (*Heliothis virescens*) larvae damaged control leaf disks, whereas, transgenic leaf disks showed no feeding damage after 4 days (Fig. 1a, b). The frequency of transformants was 85% (17 out of 20) for tobacco and 15% (6 out of 40) for cotton.

The rice calli derived from 11 out of 40 (27.5%) rice shoot apices produced ppt-resistant callus pieces (Fig. 1c). Shoot apex-derived, ppt-resistant calli and control calli on selection medium are shown in Fig. 1d. Table 1 summarizes the results of phenotype expression of herbicide and insect resistance.

To demonstrate the presence of transformed foreign DNA, we performed a slot-blot analysis on the genomic DNA from 40 ppt-resistant regenerated cotton. Six plants (phenotype positive for insect resistance) showed hybridization to the *pat* probe, whereas, no hybridization was detected in DNA from 34 other plants (Fig. 1e, f).

Fig. 1a-h Phenotype expression of herbicide- and insect-resistant plants and DNA hybridization analysis of ppt-resistant cotton plants. **a** Insect feeding bioassay of tobacco leaf disks. Control (*upper right and lower center*) leaf disks were highly damaged by the tobacco budworm (*Heliothis virescens*) larvae, whereas transgenic leaf disks showed no feeding damage after 4 days. **b** Insect feeding bioassay of cotton. Controls (*left*) were damaged by the tobacco budworm larvae, whereas transgenic leaf disks showed no feeding damage after 4 days. **c** Shoot apices-derived calli of rice at 5 weeks. Isolated shoot apices were cultured on a callus induction medium after cocultivation with *A. tumefaciens* LBA4404 (pAGM281). **d** Shoot apex-derived ppt-resistant calli (*right*) and control calli (*left*) on selection medium (4 mg l^{-1} ppt) after 6 weeks of selection. The ppt-resistant calli were obtained by three rounds of selection with 6 weeks of selection for each round. **e, f** Slot-blot analysis of genomic DNA from ppt-resistant regenerated cotton. Ten micrograms of genomic DNA was loaded in each slot and hybridized with the *pat* probe. Six plants showed positive hybrydization. **g** Rice shoot apices cultured on medium containing 4 mg l^{-1} ppt with *A. tumefaciens* LBA4404 (pAGM281 + *virG*) cocultivation (*right*) and without cocultivation (*left*). The photograph was taken after 6 weeks of selection. **h** Cotton shoot apices cultured on medium containing 2.5 mg l^{-1} ppt with *A. tumefaciens* LBA4404 (pAGM281 + *virE*/*virG*)cocultivation (*right*) and without cocultivation (*left*). The photograph was taken after 8 weeks of selection

Effect of additional *vir* genes and T-DNA size on tobacco leaf disk transformation

Table 2 summarizes the results of additional *vir* genes and T-DNA size on tobacco leaf disk transformation. The transformation efficiency of *A. tumefaciens* containing pAGM281 (8.4-kb T-DNA) was 48%, whereas *A. tumefaciens* containing pAGM277 (4.3-kb T-DNA) had

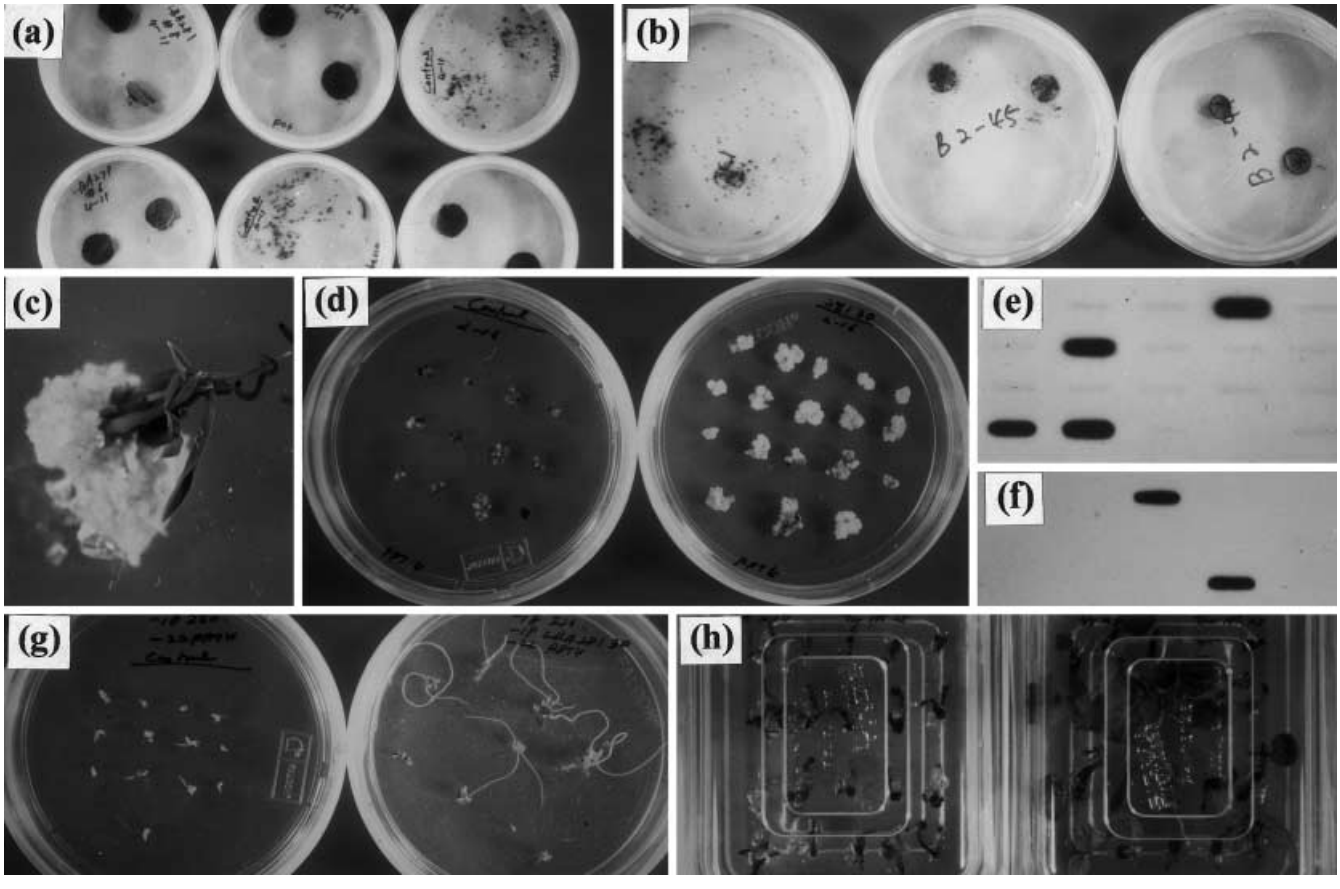

Table 1 Percentage of tobacco, cotton, and rice plants surviving selection on ppt and expressing the foreign gene phenotype

Explants	Number of tested plants[a]	Number of phenotype-expressing plants	Percentage
Tobacco leaf disks	20	17	85.0
Cotton shoot apices	40	6	15.0
Rice shoot apices	40	11	27.5

[a] Plants regenerated at selection levels that completely killed controls

Table 2 Effect of *vir* genes and T-DNA size on transformation of tobacco leaf disks

Vectors+*vir* genes	Number of leaf disks with ppt-resistant regenerated plants/number of leaf disks inoculated (%)[a]	Regenerated plants per disk[b]
pAGM281[c]	12/25 (48±11) B	1–3
pAGM277[d]	19/25 (76±17) A	2–3
pAGM281+*virG*	23/25 (92±11) A	2–4
pAGM281+*virG*N54D	23/25 (92±11) A	2–4
pAGM281+*virE*	20/25 (80±20) A	2–3
pAGM281+*virG*/*virE*	25/25 (100±0) A	2–5
Control	0/25 (0)	0

[a] One petri dish containing five disks was considered to be a replication. Different letters (A, B) indicate significant differences at $P<0.01$ by Duncan's New Multiple Range Test
[b] Disk with ppt-resistant regenerated plants
[c] T-DNA size is 8.4 kb
[d] T-DNA size is 4.3 kb

Table 3 Effect of *vir* genes and T-DNA size on transformation of cotton and rice shoot apices

Vectors±*vir* genes	Number of ppt-resistant shoot apices/number of shoot apices inoculated[a]		Percentage[b]	
	Cotton	Rice	Cotton	Rice
pAGM281	8/152	30/280	5.3±1.1 D	10.7+1.2 C
pAGM277	20/199	119/499	10.1±1.4 C	23.8±2.5 B
pAGM281+*virG*	19/125	61/201	15.2±2.5 AB	30.3±1.3 B
pAGM281+*virG*N54D	36/204	87/669	17.6±1.8 A	13.0±2.6 C
pAGM281+*virE*	15/126	87/201	11.9±3.1 BC	43.3±6.4 A
pAGM281+*virG*/*virE*	25/130	85/203	19.2±2.6 A	41.9±3.9 A
Control	0/135	0/516	0.0	0.0

[a] Total numbers of at least three independent experiments
[b] Different letters (A, B, C, D) indicate significant differences at P <0.01 by Duncan's New Multiple Range Test

a 1.6-fold increase (76%) in transformation efficiency. A significant enhancement of transformation efficiency was observed when strains with pAGM281 contained additional *vir* genes. Both *virG* and *virG*N54D led to a similar increase (92%), while *virE*/*virG* gave the highest increase (100%) in transformation efficiency. In addition, these *vir* genes increased the number of ppt-resistant regenerated plants per disk (Table 2). An 80% transformation efficiency resulted with *virE* alone.

Effect of additional *vir* genes and T-DNA size on transformation of cotton and rice shoot apices

Table 3 summarizes the results of additional *vir* genes and T-DNA size on cotton and rice shoot apex transformation. Transformation efficiency was evaluated on the basis of survival rates of cocultivated shoot apices on selection medium (Fig. 1g, h). In both cotton and rice, *A. tumefaciens* containing pAGM277 (4.3-kb T-DNA) showed a twofold increase in transformation efficiency compared with *A. tumefaciens* containing pAGM281 (8.3-kb T-DNA). However, a more dramatic enhancement of transformation efficiency resulted when *A. tumefaciens* (with pAGM281) contained additional *vir* genes.

In both cotton and rice, additional *virG* showed approximately a threefold increase in transformation efficiency (Table 3). *VirG*N54D increased the transformation efficiency of cotton threefold, whereas no increase was observed in rice. When additional *virE* was used for infection, a twofold increase in transformation efficiency of cotton and a fourfold increase in rice were observed. Additional *virE*/*virG* resulted in the highest increase (fourfold) in transformation efficiency of both cotton and rice (*virE* was equally effective in rice). These results were consistent with the data from tobacco leaf disk transformation.

To confirm the effect of additional *virE*/*virG* on transformation of cotton shoot apex, we observed the number of fluorescent spots in cotton shoot apices using GFP

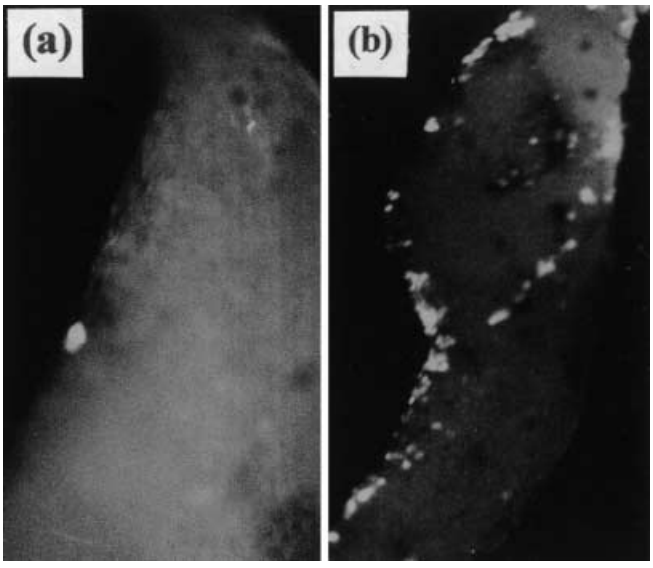

Fig. 2a, b Fluorescence microscopy image of cotton cotyledon edges 2 days after the 3 days of cocultivation. **a** Cotyledon edge with only one fluorescent spot on the edge. No *virE*/*virG* plasmid was used. Magnification: 100×, 8 s exposure time. **b** Piece of cotyledon excised from the shoot apex showing a higher number of fluorescent spots. The *virE*/*virG* plasmid was used. Magnification: 25×, 30 s exposure time

expression. When the strain with pBIN m-gfp5-ER containing additional *virE*/*virG* was used for infection, the number of fluorescent spots per area was visibly increased (Fig. 2).

Discussion

To date, various factors affecting the efficiency of *Agrobacterium*-mediated transformation have been investigated. These include cocultivation duration, the types and developmental stages of the tissues, the addition of phenolic compounds, vector constructs, *A. tumefaciens* strains, temperature, the concentration of *A. tumefaciens*, and composition of the medium.

This paper demonstrates that shorter T-DNA or additional copies of *virG* and *virE* genes in *A. tumefaciens* enhance the transformation efficiency in tobacco, cotton, and rice. While *VirE* as well as *virG* or *virG*N54D showed an enhancement in transformation efficiency in all three plant species, *virE*/*virG* resulted in the most significant enhancement. In rice, *virE* was equally effective. Additional *virG* and *virG*N54D copies both led to a similar increase of transformation efficiency in tobacco and cotton, while different results were obtained in rice. Additional *virG* increased transformation efficiency in rice, whereas no increase was observed when *virG*N54D was used. Previous studies related to the *virG* or *virG*N54D genes showed that the *vir* gene increased transformation efficiency (Liu et al. 1992; Hansen et al. 1994). Hansen et al. (1994) found that additional *virG*N54D rendered *A. tumefaciens* LBA4404 highly infectious on maize. This study showed that *virG*N54D did not enhance transformation efficiency in rice.

Earlier reports (Liu et al. 1992; Hansen et al. 1994; Hiei et al. 1994) also showed an enhancement of *Agrobacterium*-mediated transformation efficiency by additional copies of *virG* genes. Hansen et al. (1997) demonstrated that transformation efficiency was doubled with *virE2* in direct gene transfer to maize protoplasts. In addition, when *virE2* was present, the left border region (usually selectable marker gene location) suffered very little or no deletion. Hamilton (1997) suggested that the additional *virE2* gene product might enhance the transfer of very large T-DNAs. VirE2 protein protects the single-stranded T-DNA from either exonuclease or endonuclease (Citovsky et al. 1989; Rossi et al. 1996) activity and contains a nuclear localization signal involved in nuclear transport of the T-DNA into the plant nucleus (Zupan et al. 1996).

Generally, additional *virE* gene has not been necessary for T-DNA transfer because the VirE2 protein is able to coat T-DNA (below approx. 25 kb). Our results show that *A. tumefaciens* containing pAGM277 (4.3-kb T-DNA) has a higher transformation efficiency than *A. tumefaciens* containing pAGM281 (8.4-kb T-DNA) in tobacco, cotton, and rice. Moreover, transformation efficiency enhancement in all three plant species was observed when additional *virE* was added to *A. tumefaciens* containing pAGM281. The critical role of additional *virE* copies in the enhancement of transformation efficiency is not known at this time. Nevertheless, the use of additional *virE* gene copies plays an important role in enhancing transformation efficiency. Overall, the *virE*/*virG* plasmid resulted in the most significant increase in transformation frequency.

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