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GENETIC TRANSFORMATION AND HYBRIDIZATION

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High-efficiency gene transfer to recalcitrant plants by *Agrobacterium tumefaciens*

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Abstract *Agrobacterium tumefaciens* efficiently transforms most plants. A few dicotyledonous plants and most monocotyledonous plants are, however, recalcitrant to *A. tumefaciens* infection. We investigated whether the constitutive synthesis of a high level of the T-strand DNA intermediate can improve the transformation efficiency of plants. We previously described a mutation in the *vir* gene regulator *virG*, *virGN54D*, that allows constitutive expression of the *vir* genes. We also described the isolation of a mutant plasmid that is present at a significantly high level in *A. tumefaciens*. The two mutations were combined to produce an *A. tumefaciens* strain that synthesizes a high level of T-strand DNA in an inducer-independent manner. DNA transfer efficiency of the mutant was measured by monitoring β-glucuronidase (GUS) expression in a transient transfer assay. A significant increase in the efficiency of DNA transfer to both rice and soybean was observed with the double mutant. The presence of *virGN54D* had a major positive effect on transformation efficiency.

Keywords *Agrobacterium tumefaciens* · Plant transformation · Rice · Soybean · GUS gene expression

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Introduction

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 (Vasil 1996), although transformation is highly dependent upon species, genotype, competency of the target plant tissue, host recognition and other factors (Hiei et al. 1994, 1997; Ishida et al. 1996; Toki 1997). A plant transformation method based on *A. tumefaciens*-mediated gene transfer is highly desirable because in most cases a single copy of the transferred (T-) DNA is found to be integrated in the plant genome. Only occasionally is some rearrangement of the introduced DNA observed. In contrast, DNA rearrangement of the introduced DNA is a major problem in the direct DNA transfer methods (Pawlowki and Somers 1996). The simplicity of the *Agrobacterium*-based transformation method is an added advantage (Horsch et al. 1985; Chan et al. 1992; Hiei et al. 1997; Trick and Finer 1998).

The T-DNA is encoded in a large tumor-inducing (Ti-) plasmid and is defined by a conserved 25-bp border sequence found at its ends. The border sequence is the only *cis*- or *trans*-acting element essential for DNA transfer (Wang et al. 1984). The ability to replace the intervening sequence has made *A. tumefaciens* the most widely used system of gene delivery to plants. Genes essential for DNA transfer are encoded within the virulence (*vir*) region of the Ti-plasmid (reviewed in Das 1998). The *vir* gene products catalyze

the processing of the Ti-plasmid to produce a single-stranded T-strand DNA that is the intermediate in DNA transfer. The T-strand DNA is transferred to the plant cell presumably through a transport pore composed of the VirB proteins (reviewed in Christie 1997). Under normal induction conditions the *vir* genes are not expressed at their highest levels. An increase in the copy number of *virG* resulted in an increase in *vir* gene expression (Rogowsky et al. 1987). The increase in gene expression is presumably responsible for the higher efficiency of DNA transfer to plants observed in the presence of *virG* on a multicopy plasmid (Liu et al. 1992; Hansen et al. 1994).

Transcription of the *vir* genes is regulated by *virA* and *virG* (reviewed in Johnson and Das 1998). In response to a phenolic inducer(s) produced by plants, VirA phosphorylates itself and transfers the phosphate to VirG. Phosphorylated VirG, a transcriptional activator, activates transcription of all the *vir* genes. We have identified two *virG* mutants that function in an inducer-independent manner (Pazour et al. 1992). One, *virGN54D*, has an asparagine to aspartic acid substitution at position 54 and the other, *virGI106L*, has an isoleucine to leucine substitution at position 106. Both mutants, when expressed from a high-copy plasmid, allow a very high level of *vir* gene expression (Gubba et al. 1995). The commonly used wide-host-range plasmid vector, pRK2, has a relatively low-copy number in *A. tumefaciens*. Das and Xie (1995) described the isolation of RK2 mutants that are present at a three- to sevenfold higher level in *A. tumefaciens*. The incorporation of both the copy-up mutant and the *virG* constitutive mutant led to a very high (approx. tenfold higher than that in acetosyringone or plant-induced bacterium) level of *vir* gene expression (Gubba et al. 1995).

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 *A. tumefaciens*. 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. Studies of Hansen et al. (1994) support this conclusion. That study demonstrated that constitutive expression of the *vir* genes led to a significant increase in the transformation efficiency of *A. tumefaciens* and, more importantly, extended the host-range of an *A. tumefaciens* strain. Almost quantitative transformation of cotton and tobacco was obtained when the transforming bacterium harbored a plasmid containing *virGN54D*. Gene transfer to maize by *A. tumefaciens* A348 that harbors an octopine type Ti-plasmid is extremely inefficient (Grimsley et al. 1987; Boulton et al. 1989). The introduction of *virGN54D*, and not *virG*, to this strain converted *A. tumefaciens* A348 to a strain highly efficient in DNA transfer to maize, indicating that the level of *vir* gene expression plays a very important role in the virulence of the bacterium (Hansen et al. 1994). The *virGN54D* mutant supports constitutive expression of the *vir* genes, and its overexpression led to a large increase in the level of *vir* gene expression (Gubba et al. 1995). In the study reported here we investigated whether the increased level of *vir* gene expression can further improve the DNA transfer property of the bacterium. Our results demonstrate that a significant increase in DNA transfer to rice and soybean can be achieved by using the modified *A. tumefaciens* strains.

Materials and methods

Chemicals and enzymes

Carbenicillin, kanamycin, tetracycline, morpholine-ethanesulfonic acid (MES), X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronide), 4-MUG (4-methylumbelliferyl-β-d-glucuronide), 2,4-dichloro phenoxyaceticacid (2,4-D), kinetin (6-furfuryl aminopurine) and α-naphthaleneaceticacid (NAA) were purchased from Sigma Chemical (St. Louis, Mo.). Restriction and modification enzymes were purchased from New England Biolabs, Beverly, Mass.

Plant materials and growth medium

Rice (*Oryza indica*) seeds were kindly provided by Dr. B. Lockhart, University of Minnesota and Dr. Sampa Das, Bose Institute, Calcutta, India. Murashige and Skoog (MS) (1962) medium was used for rice and tobacco germination, and N6 medium (Chu et al. 1975) was used for rice cocultivation. For soybean (*Glycine max* Merrill) germination and cocultivation, B5 medium (Gamborg et al. 1968) was employed.

Seed germination

Rice seeds were dehusked, surface-sterilized with 70% ethanol for 30 s, rinsed with sterile water and sterilized again with 20% Chlorax bleach for 20 min. After being rinsed three times with water, the seeds were soaked in water for at least 1 h before being placed on germination medium. The seeds were cultured in the dark on MS basal salt solid medium supplemented with 2 mg/l 2,4-D and 0.05 mg/l NAA; they were harvested 3–5 days after germination.

Dry soybean seeds were sterilized overnight with chlorine gas released by mixing 3.3 ml 12 *N* HCl with 100 ml Chlorax in a 250-ml beaker placed inside a desiccator in a fume hood. Sterilized seeds were left at room temperature for at least 1 day before being placed on soybean germination medium. Seeds were allowed to germinate for 5 days under light conditions and then harvested for cocultivation with *A. tumefaciens*.

Plasmids and strains

The plant transformation vectors contained a β-glucuronidase (GUS) screenable marker gene and kanamycin resistance selectable marker gene. A chimeric *CaMV-GUS-nos* gene that contains the ST-LS1 intron (Vancanneyt et al. 1990) was isolated as an approximately 3-kb *Eco*RI-*Hin*dIII fragment, polished and cloned into the filled-in *Bam*HI site of the plasmid pAD1310 to construct plasmid pAD1312. Plasmid pAD1310 is a pUC118 derivative that contains the left and right border regions of the pTiA6 left T-DNA (Abuodeh et al. 2000). A 6.5-kb *Hin*dIII fragment containing the T-DNA of plasmid pAD1312 was cloned into plasmids pAD1314, 1307 and 1313 to construct plasmids pAD1320,

1322 and 1326, respectively. Plasmids pAD1307, 1313 and 1314 are wide-host-range plasmids derived from pRK2. Plasmid pAD1313 contains the wild-type pTiA6 *virG,* while the other two contain the constitutive mutant *virGN54D*. Plasmid pAD1307 harbors the RK2 mutant 75Δ2 (Das and Xie 1995). A 2.3-kb *Eco*RI-*Hin*dIII fragment containing a chimeric *CaMV-nptII-nos* gene was cloned into the T-DNA of plasmids pAD1320, 1322 and 1326 to construct plasmids pAD1338, 1339 and 1340, respectively. The plasmids were introduced into *A. tumefaciens* EHA101 by electroporation (Mersereau et al. 1990) to construct *A. tumefaciens* AD690, 691 and 692, respectively.

Bacterial growth and cocultivation

A. tumefaciens strains were grown in AB liquid medium containing 60 μg/ml carbenicillin and 10 μg/ml tetracycline at 30 °C (Pazour et al. 1992). Cells were diluted 1:20 in AB MES medium (pH 5.5) with the antibiotics and grown overnight at 25 °C. Bacteria were collected by centrifugation at 5,000 *g* for 10 min and adjusted to an $A_{600}=1$ in plant cocultivation medium.

Cocultivation of rice and *A. tumefaciens* was carried out for 3 days at 25 °C in the dark. The young shoots (5–10 mm) of newly germinated rice seedlings were cut into 3- to -5-mm-long sections on wet-sterile filter paper and immediately soaked in the *A. tumefaciens* suspension for 5–10 min. The *A. tumefaciens*-treated tissues were blot-dried on sterilized filter paper. For soybean cocultivation, explants from different segments of a germinated seedling were isolated (Fig. 3) and infected with *A. tumefaciens* for 15–30 min. The explants were placed on Whatman no.1 paper placed on solid cocultivation medium. The cotyledon node explants were placed upside down. Cocultivation was performed at 22 °C under light conditions for 3–5 days.

GUS activity assay

The histochemical assay for GUS gene expression was performed by established methods (Jefferson 1987; Kosugi et al. 1990). Following cocultivation, tissues were harvested for GUS staining. The putative transgenic explants were incubated overnight in a solution containing 25 mg/l X-gluc, 10 m*M* EDTA, 100 m*M* NaH_2PO_4, 0.1% Triton X-100 and 50% methanol, pH 8.0) at 37 °C. The number of tissues that stained blue were counted.

GUS enzyme activity was assayed as described by Jefferson (1987). At least two replicates of each sample were assayed, and the experiments were repeated twice. Tissue (100 mg) was homogenized with 500 μl GUS extraction buffer (50 m *M* sodium phosphate, pH 7.0, 10 m*M* EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 m*M* 2-mercaptoethanol). GUS activity in the extract was assayed in 200 μl of freshly prepared GUS assay buffer (35.2 mg of 4-MUG in GUS extraction buffer) at 37 °C for 30–90 min. The reaction was stopped by adding 0.9 ml of 0.2 *M* sodium carbonate. To suppress endogenous β-glucuronidase activity, we added 20% methanol to the reaction buffer.

Results and discussion

Plant transformation vector

We constructed a novel transformation vector, plasmid pAD1339, that has two unique features (Fig. 1). First, this plasmid allows inducer-independent expression of the *vir* genes because it contains a *vir* gene constitutive mutant *virGN54D* (Pazour et al. 1992). Second, it expresses the *vir* genes at a very high level because it contains a mutation that increases plasmid copy

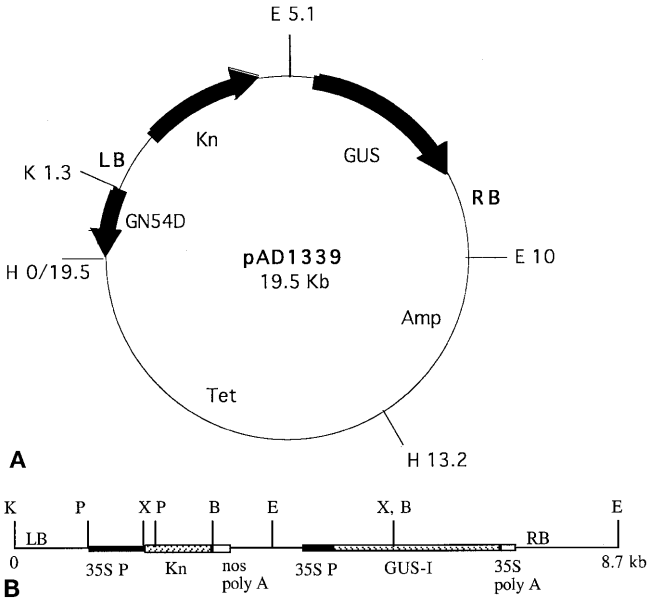

Fig. 1A,B A map of plasmid pAD1339 and its T-DNA. **A** Circular map of pAD1339. The location of *virGN54D*, GUS and the kanamycin resistance genes (*Kn*) are shown. *LB* and *RB* The left and right border sequences, respectively. The plasmid confers resistance to tetracycline and ampicillin. **B** A linear map of the T-DNA. The relative position of the kanamycin resistance gene (*Kn*) and the intron-containing GUS gene (*GUS-I*) are shown. Both genes are under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*35S P*). The poly-adenylation sequences are from the pTi nopaline synthetase (*nos*) gene or CaMV. Sites for the restriction endonucleases *Bam*HI (*B*), *Eco*RI (*E*), *Hin*dIII (*H*), *Kpn*I (*K*), *Pst*I (*P*) and *Xba*I (*X*) are shown

number in *A. tumefaciens* (Das and Xie 1995; Gubba et al. 1995). The T-DNA in the plasmid contains an intron containing the GUS gene (Vancanneyt et al. 1990) and a neomycin phospho-transferase gene (Fig. 1B). Both genes are under the control of the cauliflower mosaic virus 35S promoter. The transformation efficiency of *A. tumefaciens* harboring plasmid pAD1339 was compared to strains expressing either the wild-type *virG* or the *virGN54D* mutant on a low-copy plasmid. Rice and soybean were chosen as the host plant because these plants are relatively difficult to transform. Rice, a very important monocotyledonous crop plant, is relatively recalcitrant to *A. tumefaciens*-mediated transformation. Although a high transformation frequency of *Japonica* rice has been achieved in recent years, success in the genetic transformation of *Indica* rice has been very limited (Chan et al. 1992; Li et al. 1992; Rashid et al. 1996; Hiei et al. 1997). Soybean, a dicotyledonous species that has proven to be quite recalcitrant to regeneration from tissues, has limited susceptibility to infection by *A. tumefaciens* and transformation is restricted to non-regenerable tissues (Hinchee et al. 1988; Parrott et al. 1989; Hildebrand et al. 1991; Moore and Collins 1993; Donaldson and Simmonds 2000).

To study gene transfer we used transient expression of the reporter GUS gene as an assay. The major advantage of using transient expression as an assay is

that it reports gene activity within a few hours or days after the DNA is introduced into the plant cells. Since the majority of the transferred gene (DNA) remains extra-chromosomal during the time course of a transient gene expression assay (Dekeyser et al. 1990), analysis of gene expression is not confounded by influences caused by chromosomal DNA adjacent to the sites of an integrated gene. This assay monitors the amount of DNA delivered to the plant cell, a more accurate reflection of the primary function of the *vir* genes. The integration of the donated DNA involves other cellular functions. The host-specific difference in these functions will have a role in the stable transfer of DNA.

Gene transfer to rice

The effect of *vir* gene expression on DNA transfer to rice (*Oryza sativa* L.) was investigated by analyzing GUS expression following infection with *A. tumefaciens* expressing different levels of *virG*. Four cultivars of *Indica* rice, Ratna, Indica, Miller and FK135, were used for infection. In the presence of extra copies of *virG*, about 40–50% of the tissues stained blue in the presence of the substrate X-gluc, indicating a reasonable frequency of DNA transfer (Fig. 2A, open bars). The efficiency of DNA transfer was found to be only 5–25% in the absence of extra copies of *virG* (Liu et al. 1992). The presence of the *vir* gene constitutive mutant *virGN54D* in the transforming strain *A. tumefaciens* AD690 led to a significant increase in the efficiency of DNA transfer. The transformation frequency increased to about 75% in all cultivars. Near quantitative transformation (an efficiency of 86–90% ± 4–10%) was achieved with the super-mutant *A. tumefaciens* AD691, the strain that harbors both the high-copy mutant and *virGN54D* (dark bars). These results demonstrate that quantitative transformation of rice can be achieved using a strain that supports a high level of constitutive *vir* gene expression and that the inducer-independent expression of the *vir* genes leads to a significant increase in the transformation efficiency of *A. tumefaciens*.

Overexpression of the *vir* gene not only affected the transformation efficiency but also affected the degree of transformation. For determining transformation efficiency, we counted each tissue that stained blue as a single transformant irrespective of the number of blue spots in the tissue. Rice tissues transformed with the two mutant *A. tumefaciens*, AD690 and AD 691, had a much higher number of blue spots per tissue than tissues transformed with the strain expressing the wild-type *virG*. In addition the three strains differed in their degree of blue staining, the indicator of a GUS-positive phenotype. While the blue stains were spotty and punctuated throughout in transformants obtained from infection with the wild-type strain, a larger area of blue to dark-blue staining was observed in transformants generated from the two mutant strains (data not shown). The difference in the degree of staining indicates that the mutants delivered a higher amount of DNA to the plant cell. To obtain a better estimate of this difference we quantified GUS expression in the transformants. In all cultivars except Miller, a two-fold increase in the specific activity of β-glucuronidase was observed in transformants obtained from infection with the super-mutant strain AD691 (Fig. 2B, bars 3, 6, 9, 12). A modest 37% increase in GUS specific activity was observed with cv. Miller. This increase in transformation efficiency was due to the effect of both the *virGN54D* mutation and the higher plasmid copy number. A 30–54% increase in GUS specific activity was due to the *virGN54D* mutation (bars 2, 5, 8, 11).

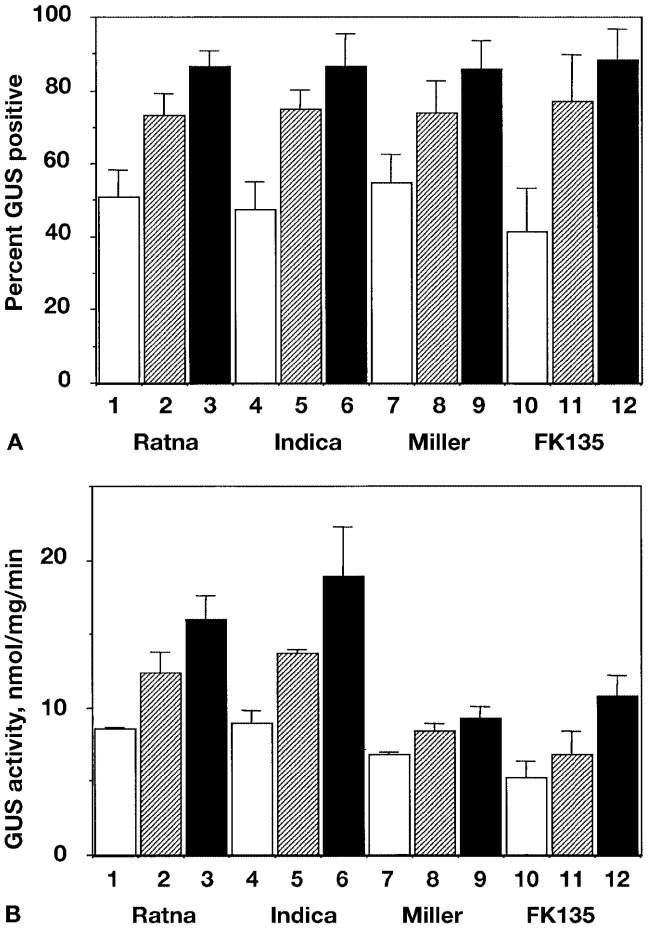

Fig. 2A,B *Agrobacterium*-mediated gene transfer to rice. Four cultivars of rice were transformed with *A. tumefaciens* AD692 (*bars 1, 4, 7, 10*), AD690 (*2, 5, 8, 11*) or AD691 (*3, 6, 9, 12*), as indicated in the Materials and methods. **A** Tissues were tested for GUS expression by the histochemical assay (Jefferson 1987), and those that stained blue were counted. **B** The level of GUS expression was quantified by enzyme assays

Gene transfer to soybean

DNA transfer to soybean was monitored on seedlings grown for 5 days after germination. The germinated

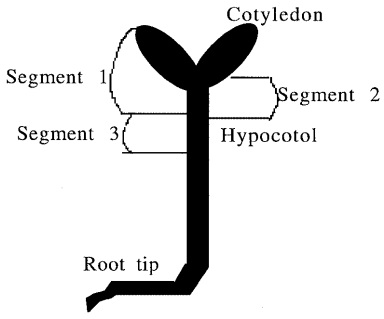

Fig. 3 Schematics of a soybean seedling. Different segments of a soybean seedling used in the transformation experiments are shown

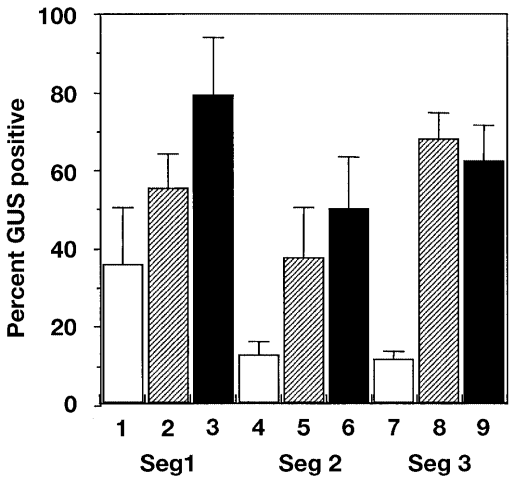

Fig. 4 Transformation of soybean by *A. tumefaciens*. The three segments of soybean seedlings (Fig. 3) were cocultivated with *A. tumefaciens* AD692 (*bars 1, 4, 7*), AD690 (*2, 5, 8*) or AD691 (*3, 6, 9*). The tissues were scored for GUS expression as described in the Materials and methods

seedlings were cut into sections (Fig. 3), infected with *A. tumefaciens* and assayed for DNA transfer by assaying GUS gene expression. Sections from the root tips were completely recalcitrant to infection with all three *A. tumefaciens* strains, indicating that the root tips was not amenable to transformation with the existing strains. The other three tissue segments tested, intact cotyledon plus a segment of the hypocotol (segment 1), part of cotyledon plus a segment of the hypocotol (segment 2) and the adjacent hypocotol (segment 3), were amenable to transformation, although they varied considerably in their sensitivity to *A. tumefaciens* (Fig. 4). While segment 1 could be transformed at a low frequency with the wild-type strain, segments 2 and 3 were relatively recalcitrant. The transformation efficiency of segment 1 increased by 126% when both mutants were incorporated into the transforming strain. About half of the increase was due to the *virGN54D* mutation. More dramatic was the effect of the mutations when the other two segments were analyzed. With both segments a transformation frequency of 10–12% was observed with the wild-type strain. Incorporation of both *virGN54D* and the copy-number mutant led to a 317% and 518% increase in the transformation frequency of segments 2 and 3, respectively. A significant amount of the increase was due to the effect of the *virGN54D* mutation. These results demonstrate that tissues recalcitrant to *A. tumefaciens* infection can be made amenable to transformation by a high level of inducer-independent *vir* gene expression.

Only a small population of cells can lead to the regeneration of a soybean plant. Most plants can be regenerated from cotyledonary nodes. For the formation of transgenic soybean plants cells in these regions need to be targeted for transformation. Therefore, to transform soybean successfully the transformation method should be able to deliver more DNA to the cotyledonary nodes. In the transformation experiment described above we analyzed the GUS-positive phenotype (blue spots) at the nodes and the base-ends of the sections (Table 1). *A. tumefaciens* AD691, the most efficient strain, led to a three- to fivefold increase in DNA transfer to the nodes of segments 1 and 2 as compared to the wild-type strain. These results support our previous conclusion that recalcitrant cells can be targeted for transformation by modulating the level of *vir* gene expression. It should be noted that the super-infective phenotype requires an inducer independent expression of the *vir* genes. The second hypocotol

Table 1 GUS-positive phenotype of soybean cotyledon nodes and hypocotyl base-ends

Explant type	Strain	*virG* genotype	Number of explants	Total no. of GUS-positive blue spots	Percentage of blue spots at:	
					Nodes	Base-ends
Segment 1	AD692	wt	66	46	11	89
	AD690	mut	61	81	19	81
	AD691	mut	61	159	29	71
Segment 2	AD692	wt	66	54	6	94
	AD690	mut	66	65	17	83
	AD691	mut	66	107	33	67
Segment 3	AD692	wt	66	21	0	100
	AD690	mut	60	97	0	100
	AD691	mut	43	176	3	97

segment, segment 3, produced a GUS-positive phenotype only at the base-ends with all three strains.

Plant transformation by *A. tumefaciens* requires the presence of a *vir* gene inducer in the plant cell (Stachel et al. 1985). The ability of *A. tumefaciens* to transform both *Indica* rice and soybean, plants relatively difficult to transform, indicates that both plants produce a *vir* gene inducer(s). However, the constitutive high level of *vir* gene expression from the supervirulent strain used in this study led to a considerable increase in DNA transfer efficiency to both plants. Our results indicate that one reason for the low transformation frequency of both plant species was the low induction of *vir* gene expression in the infecting bacteria. An increase in *vir* gene expression in conjunction with their constitutive expression led to very high level of transformation of both plant species. In a previous study we demonstrated that the constitutive expression of the *vir* genes was highly effective in increasing the transformation efficiency of tobacco, cotton and maize (Hansen et al. 1994). Therefore, this approach is likely to be effective for the transformation of a large variety, if not all, of the plant species. The use of the endogenous *vir* gene inducers is not a very effective approach for achieving high transformation efficiency of recalcitrant plants.

Studies using the various cultivars of rice as well as tissue segments of soybean have demonstrated that the super virulent strain is much more effective in delivering DNA to the plant cell. In these studies a two- to sevenfold increase in the number of GUS-positive tissues was observed, and in several cases a significant increase in the GUS enzyme activity was found. The latter can result only from an increase in the level of DNA transferred to individual tissue segments. The rice cultivars responded differently to the *A. tumefaciens* strains. While all cultivars were transformed quantitatively by the supervirulent strain, significant differences in the specific activity of GUS were observed between the cultivars. These results indicate that during transformation a different amount of DNA is transferred to each species.

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References

Abuodeh RO, Orbach MJ, Mandel MA, Das A, Galgiani JN (2000) Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. J Infect Dis 181:2106–2110

Boulton M, Buchholz W, Marks M, Markham P, Davies J (1989) Specificity of *Agrobacterium*-mediated delivery of maize streak virus DNA to members of the gramineae. Plant Mol Biol 12:31–40

Chan MT, Lee TM, Chang HH (1992) Transformation of indica rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. Plant Cell Physiol 33:577–583

Christie P (1997) *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. J Bacteriol 179:3085–3094

Chu CC, Wang CS, Sun CC, Hsu C, Yin KC, Chu CY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Das A (1998) DNA transfer from *Agrobacterium* to plant cells in crown gall tumor disease. In: Biswas BB, Das HK (eds) Subcellular biochemistry: plant microbe interactions. Plenum, London, pp 343–363

Das A, Xie Y-H (1995) Replication of a broad-host-range plasmid RK2:isolation and characterization of a spontaneous deletion mutant that can replicate in *Agrobacterium tumefaciens* but not in *Escherichia coli*. Mol Gen Genet 246:309–315

Dekeyser RA, Claes B, De Rycke RMU, Habets ME, van Montagu MC, Caplan AB (1990) Transient gene expression in intact and organized rice tissues. Plant Cell 2:591–602

Donaldson PA, Simmonds DH (2000) Susceptibility to *Agrobacterium tumefaciens* and cotyledonary node transformation in short-season soybean. Plant Cell Rep 19:478–484

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:152–158

Grimsley N, Hohn T, Davies J, Hohn B (l987) Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. Nature 325:177–179

Gubba S, Xie Y-H, Das A (1995) Regulation of *Agrobacterium tumefaciens* virulence gene expression: isolation of a mutation that restores *vir*GD52 E function. Mol Plant Microbe Interact 8:788–791

Hansen G, Das A, Chilton MD (1994) Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*. Proc Natl Acad Sci USA 91:7603–7607

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the T-DNA. Plant J 6:271–282

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol Biol 35:205–218

Hildebrand DF, Liu W, Deng W, Grayburn WS, Collins GB (1991) Progress in biotechnological approaches in the improvement of soybean seed quality. In: Rattray JBM (ed) Biotechnology and the oils and fats industry. American Oil Chemistry Society Press, Champaign, Ill., pp 58–75

Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated gene transfer. Biotechnology 6:915–922

Horsch R, Fry J, Hoffman N, Eichholtz D, Rogers S, Fraley R (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Biotechnol 14:745–750

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Johnson T, Das A (1998) Organization and regulation of expression of the *Agrobacterium* virulence genes. In: Spaink H, Kondorosi A, Hooykaas P (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 267–279

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci 70:133–140

Li XQ, Liu CN, Ritchie ST, Peng J, Gelvin SB, Hodges TK (1992) Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. Plant Mol Biol 20:1037–1048

Liu CN, Li XQ, Gelvin SB (1992) Multiple copies of *virG* enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. Plant Mol Biol 20:1071–1087

Mersereau M, Pazour G, Das A (1990) Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene 90:149–151

Moore PJ, Collins GB (1993) Transformation in soybean (*Glycine max* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, plant protoplasts and genetic engineering, vol 4. Springer, Vienna Berlin Heidelberg, pp 228–236

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Parrott WA, Hoffman LM, Hildebrand P, Williams EG, Collins GB (1989) Recovery of primary transformants of soybean. Plant Cell Rep 7:615–617

Pawlowki WP, Somers DA (1996) Transgene inheritance in plants genetically engineered by microprojectile bombardment. Mol Biotechnol 6:17–30

Pazour GJ, Ta C, Das A (1992) Constitutive mutations of *Agrobacterium tumefaciens* transcriptional activator *virG*. J Bacteriol 174:4169–4174

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *A. tumefaciens* in *Indica* rice. Plant Cell Rep 15:727–730

Rogowsky PM, Close TJ, Chimera JA, Shaw JJ, Kado CI (1987) Regulation of *vir* genes of *A. tumefaciens tumefaciens* plasmid pTiC58. J Bacteriol 169:5101–5112

Stachel SE, Messens E, Van Montagu M, Zambryski PC (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *A. tumefaciens tumefaciens*. Nature 318:624–629

Toki S (1997) Rapid and efficient *A. tumefaciens*-mediated transformation in rice. Plant Mol Biol Rep 15:16–21

Trick HN, Finer JJ (1998) Sonication-assisted *A. tumefaciens*-mediated transformation of soybean [*Glycine max* (L.) Merrill] embryogenic suspension culture tissue. Plant Cell Rep 17:482–488

Vancanneyt G, Schmidt R, OíConnor-Sanchez A, Willmitzer L, Rocha-Sasa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *A. tumefaciens*-mediated plant transformation. Mol Gen Genet 220:245–250

Vasil IK (1996) Milestones in crop biotechnology-transgenic cassava and *Agrobacterium*-mediated transformation of maize. Nat Biotechnol 14:702–703

Wang K, Herrerra-Estrella L, Van Montagu M, Zambryski P (1984) Right 25-bp terminus sequences of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38:455–462