Constitutive expression of the virulence genes improves the efficiency of plant transformation by Agrobacterium

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ABSTRACT Inducible virulence (vir) genes of the Agrobacterium tumefaciens tumor-inducing (Ti) plasmid are under control of a two-component regulatory system. In response to environmental factors (phenolic compounds, sugars, pH) VirA protein phosphorolyses VirG, which in turn interacts with the promoters of other vir genes, causing induction. A mutation of VirG, virGN54D (which codes for a Asm-S4 → Asp amino acid change in the product), causes constitutive expression of other vir genes independent of VirA. We have investigated whether providing Agrobacterium with a plasmid containing virGN54D augments the efficiency of transfer of the T-DNA (transformed DNA). For both tobacco and cotton, we observed an enhancement of transformation efficiency when the inciting Agrobacterium strain carries the virGN54D mutation. We also tested whether supplying Agrobacterium with a similar plasmid containing wild-type VirG affects the efficiency of T-DNA transfer. An intermediate efficiency was observed when this plasmid was employed. Using a β-glucuronidase (GUS) reporter gene to assess transient expression of T-DNA after transfer to tobacco and maize tissues, we observed a higher frequency of GUS-expressing foci after inoculation with Agrobacterium strains carrying virGN54D than with Agrobacterium carrying the wild-type VirG. Gene-transfer efficiency to maize by an octopine strain was greatly improved upon introduction of virGN54D. Multiple copies of wild-type VirG were equally effective in promoting transient expression efficiency in tobacco but were virtually ineffective in maize. We propose the use of virGN54D to improve the efficiency of Agrobacterium-mediated transformation, especially for recalcitrant plant species.

Agrobacterium tumefaciens and Agrobacterium rhizogenes incite crown gall tumors and hairy root disease, respectively, on a wide range of dicotyledonous plants and gymnosperms and on some monocotyledonous plants. Virulent strains of Agrobacterium harbor a large tumor-inducing (Ti) or root-inducing (Ri) plasmid. During the process of infection at wound sites in susceptible plants, a specific segment of the plasmid, T-DNA (transformed DNA), is transferred from the bacterium to the host plant cells and is inserted into the nuclear genome. Ti plasmid T-DNA contains genes for synthesis of cytokinin and auxin, phytohormones that cause proliferation of plant cells to form the gall (reviewed in ref. 1). The transformed plant cells are also directed by T-DNA genes to produce unusual metabolites called opines, such as octopine and nopaline, that serve as specific nutrients for the inciting bacteria (2) and are often used to type Ti plasmids. T-DNA on the Ti plasmid is bordered by directly repeated 25-bp sequences that are required in cis for its excision and transfer. No other part of T-DNA is necessary for its transfer (3). Excision and transfer functions of T-DNA are mediated by virulence functions that map on the Ti plasmid outside T-DNA and are encoded by several vir genes and operons (virA, virB, virC, virD, virE, virG). Expression of the virulence genes results in the production in Agrobacterium of one or more linear copies of the lower strand of the T-DNA called “T-strands” (4, 5). The T-strand is capped at the 5' end by the virD2 gene product (6, 7) and is protected by a single-strand binding protein encoded by virE2 (8, 9). The T-strand is believed to be directed to the plant nucleus by nuclear localization sequences in VirD2 and VirE2 proteins (10–12). T-DNA integrates into the plant chromosomes at seemingly random locations by an unknown mechanism.

Virulence functions are transcriptionally regulated by a two-component gene-regulatory system that responds to the chemical environment (13). Optimal vir gene induction occurs at acidic pH and in the presence of phenolic inducers, such as acetosyringone (AS) (14), that are released by wounded plant cells. The vir gene regulatory system operates through two monocistronic virulence genes: virA and virG. The constitutively expressed virA gene produces a protein located in the inner membrane that recognizes plant wound metabolites (3). The resulting autophosphorylation of VirA protein activates the intracellular signal-transducing protein VirG by phosphorylation of an aspartic acid residue (15). The activated VirG binds to vir gene promoters at conserved vir box sequences and acts as a transcriptional activator of the virulence genes (16, 17).

Many dicot plants and gymnosperms are susceptible to Agrobacterium and are potentially transformable by Agrobacterium, although at various efficiencies. Most monocots such as maize appear to be less susceptible to Agrobacterium infection. However, synthesis of opines has been reported for young maize seedlings after Agrobacterium inoculation (18). Expression of a chimeric β-glucuronidase (GUS) gene carried by the T-DNA was also reported to be expressed in excised maize seedling apices (19) and in shoots of young maize seedlings (20). Moreover, T-DNA transfer from Agrobacterium to maize cells has been demonstrated by agrobiofication, a technique using Agrobacterium-mediated transfer of viral sequences to plant cells (21).

There may be several problematic steps that can limit the efficiency of transformation, but surely one of the most critical steps is the induction of the virulence genes. In the case of maize, which is not efficiently transformable by Agrobacterium, it has been shown that 2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one (DIMBOA), a metabolite found in maize homogenates, specifically inhibits the induction of Ti plasmid virulence genes at sublethal concentration (22). Expression of vir genes independent of environmental parameters could allow Agrobacterium to transfer T-DNA more efficiently to monocot plants such as maize. It has been shown that an increase in copy number of virA and virB has a significant effect on transformation efficiency (23).

Abbreviations: AS, acetosyringone; GUS, β-glucuronidase; MS, Murashige and Skoog medium; T-DNA, transformed DNA; Ti and Ri plasmids, tumor-inducing and root-inducing plasmids; Km, kanamycin; NptII, neomycin phosphotransferase II; DIMBOA, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3(4H)-one.

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virG genes can partially relieve the pH dependence of vir gene induction (23, 24). Recently, we have isolated a mutant of virG, virGN54D (with an altered codon that codes for a Asn-54 → Asp amino acid change in the product), that allows high-level constitutive vir gene expression in the absence of virA and chemical inducing agents (25). We present here an assessment of the effect of virGN54D on the efficiency of Agrobacterium-mediated transformation of tobacco and cotton and on the frequency of transient expression in tobacco and maize.

MATERIALS AND METHODS

Strains and Plasmids. Plasmid pAD1288 was constructed by cloning a blunt-ended 5-kb EcoR1-HindIII restriction fragment containing pRH1R origin of replication (26) into the blunt-ended HindIII site of pGPI09 (25). pGPI09 harbors a copy of the virG gene from the octopine Ti plasmid pTiA6. pAD1289 is the corresponding plasmid carrying the mutant virGN54D. The binary vector pBG5 is a pBIN19 derivative that contains a nos-NptII 2-3 nos selectable marker gene for plant transformation conferring kanamycin (Km) resistance (neomycin phosphotransferase II gene bracketed by the promoter and terminator of the nopaline synthase gene nos) and a 35S-GUS-nos gene coding for GUS under the control of the cauliflower mosaic virus 35S promoter and the nos terminator (20). This construct shows no detectable expression in Agrobacterium (20). Further details of the strains used in this study are listed in Table 1.

Media. Bacteria were cultured in YEB medium (5 g of beef extract, 1 g of yeast extract, 5 g of Bactopeptone, and 5 g of sucrose per liter, pH 7.2) with the following concentrations of selective antibiotics as appropriate for the various plasmids: ampicillin at 100 mg/liter (pAD1288 and pAD1289) or Km at 100 mg/liter (pBG5). Plant tissues were cultivated on Murashige–Skog medium (MS) (28) supplemented with Km at 25 or 100 mg/liter and cefotaxime at 500 mg/liter where indicated.

Plant Growth and Inoculation Conditions. Infection of tobacco. Transformation of Nicotiana tabacum cv. Xanthi was carried out by leaf disc inoculation (29). Tobacco leaf discs from 4- to 5-week-old axenically grown tobacco plants were imersed in Agrobacterium suspension (10⁶ cells per ml) for 15 min and plated on MS medium. After cocultivation for 2 days, leaf discs were transferred to fresh MS medium supplemented with 100 mg of Km and 500 mg of cefotaxime per liter and incubated at 25°C with a 16-hr photoperiod. Infection of tobacco. Transformation of Nicotiana tabacum cv. Xanthi was carried out by leaf disc inoculation (29). Tobacco leaf discs from 4- to 5-week-old axenically grown tobacco plants were imersed in Agrobacterium suspension (10⁶ cells per ml) for 15 min and plated on MS medium. After cocultivation for 2 days, leaf discs were transferred to fresh MS medium supplemented with 100 mg of Km and 500 mg of cefotaxime per liter and incubated at 25°C with a 16-hr photoperiod. After 3 days of cocultivation, the cotyledon tissues were placed on the same medium containing 25 mg of Km and 500 mg of cefotaxime per liter and incubated at 30°C with a 16-hr photoperiod.

Infection of maize shoots. The infection of maize shoots was performed essentially as described (20). Immature zygotic embryos (14 days after pollination; ca. 2 mm long) of the maize line A188 were picked from greenhouse-grown ears. Excised embryos were plated on MS medium with 3% sucrose and incubated at 25°C under a 16-hr photoperiod. Shoots were excised from 3-day-old seedlings by cutting at the coleoptilar node. Shoots were immersed in an Agrobacterium suspension (10⁶ cells per ml), subjected to vacuum infiltration for 10 min, and then cultured on MS medium supplemented with 100 μM AS for 3 days at 25°C under a 16-hr photoperiod. Shoots were stained for 2 days with 0.05% 5-bromo-4-chloro-3-indolyl glucuronide in 100 mM NaH₂PO₄ (pH 7). Shoots were then destained for 1 day with ethanol to remove chlorophyll before scoring blue spots.

Infection of tobacco seedlings. An overnight culture (5 ml) of Agrobacterium grown in YEB medium was washed and resuspended in 10 ml of MS medium. Axenic seed-grown tobacco plantlets of Nicotiana tabacum cv. Xanthi, grown for 12 days in a growth chamber at 26°C with a 16-hr photoperiod, were immersed in the bacterial suspension and subjected to vacuum infiltration for 15 min (12). The plantlets were then placed on MS plates. After cocultivation for 3 days in a growth chamber at 26°C, the plantlets were analyzed by histochemical GUS stain (31). The plantlets were destained with ethanol to remove chlorophyll before scoring blue spots.

RESULTS

Stable Transformation of Tobacco and Cotton. The effect of virGN54D on plant transformation was investigated by assaying transformation efficiency on tobacco and on cotton. Supplementary copies of virG or virGN54D were introduced into the desired Agrobacterium strain by introducing pAD1288 or pAD1289, respectively. All strains in addition contained plasmid pBG5 (20), a plasmid carrying a modified 35S-GUS-nos gene and the nos-NptII-nos gene as a plant selectable marker between T-DNA borders. After inoculation with these strains, plant tissues were plated on hormone-free medium containing Km to allow growth of transformed tissues only—i.e., cells that acquired hormone independence from integration of the T-DNA of pTiC58 or pTiAch5 as well as Km resistance from the expression of the NptII gene of pBG5. Transformation efficiency was measured by semiquantification of Km-resistant hormone-independent calli.

The transformation efficiency of Agrobacterium strains CS8 and Ach5 containing the various constructs was assayed on tobacco leaf discs and cotton cotyledon tissues. Experiments were performed on discs of equal size, and inoculations were done with equal concentrations of bacterial cells to compare transformation efficiency. The number of individual Km-resistant tumors was scored 2 weeks after inoculation for tobacco and 4 weeks after inoculation for cotton. Table 2 summarizes the results of three independent experiments.

Patterns for transformation of tobacco leaf discs and cotton cotyledon tissues were similar. In harmony with previous studies (32), we observed that both the octopine and the nopaline strains of Agrobacterium with pBG5 and pAD1288 carrying the wild-type virG exhibited a 2- to 3-fold increase in transformation efficiency compared to strains carrying pBG5. Agrobacterium with pAD1289 carrying virGN54D exhibited a further increase in transformation efficiency (Table 2). Both tobacco and cotton could be transformed almost quantitatively (70–93%) with either the octopine or the nopaline strain containing virGN54D. Transformation efficiency of strains with the control virG construct (pAD1288) was 50–65%.

Table 1. Agrobacterium strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Chromosomal background</th>
<th>Ti plasmid</th>
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<tbody>
<tr>
<td>CS8</td>
<td>CS8</td>
<td>pTiC58 (a nopaline-type plasmid)</td>
</tr>
<tr>
<td>Ach5</td>
<td>Ach5</td>
<td>pTiAch5 (an octopine-type plasmid)</td>
</tr>
<tr>
<td>LBA4404</td>
<td>Ach5</td>
<td>pAL4404 (a deletion mutant of pTiAch5 lacking T-DNA but having a full set of vir genes; ref. 27)</td>
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</table>
We noted an additional effect of providing Agrobacterium with the virG- or virGN54D-containing plasmid: tumors appeared at an earlier time, and the number of individual tumors per disc increased to 8–10 for tobacco and 2–3 for cotton. With the parental strains C58(pBG5) and Ach5(pBG5), only 2 or 3 tumors per disc formed on tobacco and 1 or 2 on cotton cotyledons.

Strains of Agrobacterium without the plasmid pBG5 were also used to infect cotton and tobacco tissues as described above. Infected tissues were plated on MS with or without Km. The number of tumors on MS medium observed 2 or 4 weeks after inoculation for tobacco tissues and cotton tissues, respectively, matched the frequency of Km-resistant tumors in the corresponding pBG5-containing strain (data not shown). As expected, usually no tumors were detected on MS with Km; in rare cases, however, small tumors emerged but did not grow further (data not shown).

**Transient Expression in Maize Shoots.** We investigated the effect of virG and virGN54D on transient expression efficiency in young maize and tobacco seedlings. Maize was chosen because it is relatively refractory to Agrobacterium infection, although both transient expression (20, 21) and stable transformation (18, 19) of maize by Agrobacterium have been reported. In this study, the T-DNA transfer assay relied upon expression of a chimeric GUS gene in plant tissues 3 days after inoculation. All of the strains of Agrobacterium tested contained a plasmid with a 35S-GUS gene (pBG5) (20) to allow monitoring of the transient expression of transferred genes in these plant tissues.

To determine the frequency of transient transformation of maize tissues, we used the protocol of Shen et al. (20). Shoots excised from 3-day-old seedlings grown from immature embryos of the maize line A188 were infected with Agrobacterium, and histochemical GUS assays were performed 3 days after infection. AS was included in the cocultivation agar, but bacteria were not preincubated. It has been shown that pre-treatment of dicot plant tissues with AS (33) or use of AS during cocultivation (34) improved transformation efficiency by enhancing plant cell division (33).

Results of these studies, summarized in Table 3, revealed interesting differences between the octopine and nopaline strains. In nopaline strain C58(pBG5), blue spots indicating GUS activity were observed on 36–40% of the shoots after cocultivation. The effect of adding plasmid pAD1288 (with virG) appeared nil (35–42%); however, the addition of pAD1289 (with virGN54D) to this strain led to a 2-fold increase in the percentage of shoots showing blue spots (78–83%). The impact of virGN54D was even more dramatic when the octopine strain LBA4404 was used for infection. As reported previously (20), the octopine strain LBA4404 showed a very low efficiency of DNA transfer (0–15% of shoots with blue spots). A slight increase in DNA transfer efficiency (10–25%) was observed when plasmid pAD1288 (with virG) was introduced into this strain. Strikingly, when pAD1289 containing virGN54D was introduced into this strain, 70–77% of the shoots showed GUS-positive blue spots, indicating an efficiency of DNA transfer similar to that of the nopaline strain in the presence of virGN54D.

**Transient Expression in Tobacco.** In transient expression assays with tobacco, both pAD1288 and pAD1289 led to a similar increase (2–4 fold) in DNA transfer efficiency (Table 3). C58(pBG5) produced GUS-positive blue spots in only 16–25% of tobacco seedlings. Addition of pAD1288 carrying virG increased this frequency to 66–75%; and addition of pAD1289 carrying virGN54D produced 75–83% frequency of transiently expressing plantlets. Strain LBA4404 (pBG5) produced GUS-positive blue spots 33–50% of the time. Addition of the plasmid carrying virG increased this value to 83%, while that with virGN54D gave similar results (83–91%). However, the number of GUS-positive blue spots was higher when the infecting bacteria contained virGN54D strain (Fig. 1).

To assure that these GUS-positive spots are indeed the consequence of transfer of T-DNA into the plant cells, we used as control a T-DNA transfer-deficient Agrobacterium strain containing an insertion mutation in virB. Under the experimental conditions used in our study, tobacco seedlings and maize shoots inoculated with this strain did not exhibit any blue spots (data not shown).

**DISCUSSION**

**virGN54D Improves Stable Transformation Efficiency.** In this study, plasmid pAD1289 carrying virGN54D in the

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. of tissues stained blue/total no. tested (%)</th>
<th>Maize</th>
<th>Cotton</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Exp. 3</td>
</tr>
<tr>
<td>C58 (pBG5)</td>
<td>18/50 (36)</td>
<td>21/54 (38)</td>
<td>16/40 (40)</td>
</tr>
<tr>
<td>C58 (pBG5, pAD1288)</td>
<td>15/42 (35)</td>
<td>19/48 (39)</td>
<td>17/40 (42)</td>
</tr>
<tr>
<td>C58 (pBG5, pAD1289)</td>
<td>20/24 (83)</td>
<td>25/32 (78)</td>
<td>24/30 (78)</td>
</tr>
<tr>
<td>LBA4404 (pBG5)</td>
<td>3/20 (15)</td>
<td>2/18 (11)</td>
<td>0/21 (0)</td>
</tr>
<tr>
<td>LBA4404 (pBG5, pAD1288)</td>
<td>2/20 (10)</td>
<td>5/20 (25)</td>
<td>3/18 (16)</td>
</tr>
<tr>
<td>LBA4404 (pBG5, pAD1289)</td>
<td>36/48 (77)</td>
<td>15/21 (71)</td>
<td>28/40 (70)</td>
</tr>
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</table>

Table 2. Effect of additional copies of virG (pAD1288) and of virGN54D (pAD1289) on transformation of tobacco leaf discs and cotton cotyledon segments

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. leaf discs with Km-resistant tumors/total no. tested (%)</th>
<th>Tobacco</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>C58 (pBG5)</td>
<td>16/46 (35)</td>
<td>15/41 (36)</td>
</tr>
<tr>
<td>C58 (pBG5, pAD1288)</td>
<td>47/72 (65)</td>
<td>48/80 (60)</td>
</tr>
<tr>
<td>C58 (pBG5, pAD1289)</td>
<td>61/71 (86)</td>
<td>68/80 (85)</td>
</tr>
<tr>
<td>Ach5 (pBG5)</td>
<td>10/30 (33)</td>
<td>12/30 (40)</td>
</tr>
<tr>
<td>Ach5 (pBG5, pAD1288)</td>
<td>43/70 (61)</td>
<td>38/60 (63)</td>
</tr>
<tr>
<td>Ach5 (pBG5, pAD1289)</td>
<td>61/70 (87)</td>
<td>56/60 (93)</td>
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</tbody>
</table>

Table 3. Transient GUS expression on shoots of maize seedlings and on tobacco plantlets after cocultivation with Agrobacterium and its mutants
infecting strain enhanced the efficiency of Agrobacterium-mediated transformation in both tobacco and cotton. Agrobacterium containing this mutant virG exhibits constitutive high levels of vir gene expression in the absence of virA and plant phenolic inducers (24, 25). Infection of tobacco leaf discs with a strain containing virGN54D resulted in a 2-fold increase in frequency of transformed leaf discs compared with the parental strain. In cotton cotyledon transformation, an increase of ca. 4-fold was observed. When plasmid pAD1288 containing wild-type virG was introduced into Agrobacterium as a control, an intermediate frequency of transformation was observed (1.5-fold increase for tobacco, 3-fold for cotton). These results indicate that induction of vir genes is indeed a limiting feature of tumor induction in these systems and that virGN54D can confer superior T-DNA transfer efficiency.

virGN54D Renders Octopine-Type Agrobacterium Highly Infectious on Maize. In maize, octopine-type Agrobacterium strains are much less efficient than nopaline-type strains in agroinfection (21, 35) and in producing transient expression of T-DNA (20). The inefficiency could be corrected in large part by addition of a plasmid containing the virA gene from the nopaline-type plasmid (36). The results reported here show that addition of virGN54D circumvents the problem of the octopine strain in transient infection of maize, rendering it as effective as the nopaline strain. This result is fully consistent with the interpretation (reviewed in ref. 37) that vir gene induction is indeed the problematic step for maize infection by the octopine strains.

virG Effects on Efficiency of Transient Expression. Liu et al. (32) have demonstrated that addition of a multicopy plasmid carrying virG to an Agrobacterium strain enhanced the transient transformation frequency for celery, carrot, and rice tissues. Our studies further explore the scope of this effect. In tobacco, the frequency of plants expressing GUS transiently was significantly increased by addition of pAD1288 carrying wild-type virG to the infecting strain. However, we find that provision of this plasmid to either C58- or LBA4404-derived Agrobacterium strains produces little or no increase in the frequency of transient expression in maize. One significant methodological difference is that Liu et al. (32) preinduced the Agrobacterium with AS for 24 hr before infection of plant tissues. In this study, bacteria were not preinduced, but AS was included in the cocultivation agar. It is plausible that preinduction of vir genes with AS would be especially important for maize seedling infection because

DIMBOA produced by maize can presumably inhibit induction of vir genes during bacterial invasion of the plant (22).

Role of virG in Hypervirulence. The key role of virG in determining transformation efficiency is clear from the finding that hypervirulent Agrobacterium Ti plasmid pTiBo542 has a normally virulent T-DNA (38) but a hypervirulent form of virG (39). The hypervirulence of pTiBo542 virG is mediated by higher levels of expression of vir genes after induction (40). Under optimal induction conditions, the coding region (which has two amino acid substitutions) is responsible for the hypervirulence of this gene. The constitutive virGN54D used in this study has a single amino acid substitution that maps between the pTiBo542 virG changes (39). Although a constitutive mutant version of this supervirulent virG might be expected to produce superior constitutive levels of vir gene expression, a mutation that converts Asn-54 to Asp in the pTiBo542 virG gene produced a slightly lower level of virB:lacZ expression than does the pTiA6 virGN54D (24).

Implications for Vector Design. Disarmed Ti/Ri plasmids or binary vectors have been used to transfer genes for herbicide resistance, disease resistance, and insect resistance, and, in some cases, into economically important crops (reviewed in ref. 41). In Agrobacterium strains with virGN54D, T-DNA transfer can occur immediately and thus should be less subject to adverse environmental factors. This should be especially important in recalcitrant plants, which may lack sufficient or appropriate inducer chemicals and/or, like maize (22), may produce secondary metabolites that are inhibitors of induction. Recalcitrant plants may also produce toxic compounds that reduce the Agrobacterium population before the bacteria can mount an induction and transfer T-DNA. Constitutive vir gene expression conferred by virGN54D should enable Agrobacterium to transfer T-DNA immediately with no lead time for induction. We propose that incorporation of virGN54D into transformation vectors outside T-DNA will improve the efficiency of Agrobacterium-mediated transformation. We further suggest that this stratagem may be particularly valuable for plants that appear refractory or even immune to Agrobacterium T-DNA transfer.

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