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Early Transcription of Agrobacterium T-DNA Genes in Tobacco and Maize

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We developed a sensitive procedure to investigate the kinetics of transcription of an *Agrobacterium tumefaciens* transferred (T)-DNA-encoded β-glucuronidase *gusA* (*uidA*) gene soon after infection of plant suspension culture cells. The procedure uses a reverse transcriptase-polymerase chain reaction and enables detection of *gusA* transcripts within 18 to 24 hr after cocultivation of the bacteria with either tobacco or maize cells. Detection of *gusA* transcripts depended absolutely on the intact virulence (*vir*) genes *virB*, *virD1/virD2*, and *virD4* within the bacterium. Mutations in *virC* and *virE* resulted in delayed and highly attenuated expression of the *gusA* gene. A nonpolar transposon insertion into the C-terminal coding region of *virD2* resulted in only slightly decreased production of *gusA* mRNA, although this insertion resulted in the loss of the nuclear localization sequence and the important ω region from VirD2 protein and rendered the bacterium avirulent. However, expression of *gusA* transcripts in tobacco infected by this *virD2* mutant was more transient than in cells infected by a wild-type strain. Infection of tobacco cells with an Agrobacterium strain harboring a mutant *virD2* allele from which the ω region had been deleted resulted in similar transient expression of *gusA* mRNA. These data indicate that the C-terminal nuclear localization signal of the VirD2 protein is not essential for nuclear uptake of T-DNA and further suggest that the ω domain of VirD2 may be required for efficient integration of T-DNA into the plant genome. The finding that the initial kinetics of *gusA* gene expression in maize cells are similar to those shown in infected tobacco cells but that the presence of *gusA* mRNA in maize is highly transient suggests that the block to maize transformation involves T-DNA integration and not T-DNA entry into the cell or nuclear targeting.

INTRODUCTION

During the inception of crown gall tumorigenesis, *Agrobacterium tumefaciens* processes a region of DNA (the transferred or T-DNA) from the resident tumor-inducing (Ti) plasmid and transfers this DNA to plant cells. Proteins encoded by the virulence (*vir*) region of the Ti plasmid regulate T-DNA processing and transfer. Nicking of 25-bp directly repeated T-DNA "border" sequences by the VirD2 endonuclease results in the generation of single-stranded T-DNA molecules (T strands) with which VirD2 is tightly associated at the 5′ end (Herrera-Estrella et al., 1988; Ward and Barnes, 1988; Young and Nester, 1988; Howard et al., 1989). These single-stranded DNA molecules are transferred to the plant cytoplasm (Yusibov et al., 1994), perhaps as a complex (the T complex; Howard and Citovsky, 1990) with the single-stranded DNA binding protein VirE2 (Gietl et al., 1987; Christie et al., 1988; Citovsky et al., 1988; Das, 1988). Targeting of the T-DNA to the plant nucleus may be mediated by nuclear localization sequences (NLSs) within the associated VirD2 and VirE2 proteins (Herrera-Estrella et al., 1990; Howard et al., 1992; Shurvinton et al., 1992; Tinland et al., 1992; Koukolikova-Nicola et al., 1993; Rossi et al., 1993; Citovsky et al., 1994). T-DNA molecules eventually integrate into the plant chromosomes, thereby stabilizing the oncogenes and opine biosynthesis genes encoded by the T-DNA. However, nonintegrated copies of T-DNA may persist in the nucleus for a period of time.

During the past two decades, research in a number of laboratories has resulted in a fairly detailed understanding of the early events in Agrobacterium that result in *vir* gene induction, T-DNA processing, and T-DNA transfer. Similarly, we now have a good understanding of the molecular mechanisms of T-DNA expression that result in opine biosynthesis and tumorigenesis within the plant cell (reviewed in Ream, 1989; Winans, 1992; Zambryski, 1992; Gelvin, 1993; Hooykaas and Beijersbergen, 1994; Zupan and Zambryski, 1995). Little is known, however, about the events within the plant cell that involve the targeting of T-DNA to the nucleus, its ultimate integration into plant nuclear DNA, and the early stages of T-DNA expression. To date, the earliest detectable expression of T-DNA–encoded genes

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occurs 2 days after infection. Fraley et al. (1984) first detected nopaline biosynthetic activity 2 days after infection of regenerating petunia protoplasts by Agrobacterium. Using a modified cauliflower mosaic virus 35S promoter–β-glucuronidase *gusA* chimeric gene that lacks a Shine-Dalgarno ribosome binding site (such that GUS expression is minimized in Agrobacterium), Janssen and Gardner (1989) detected GUS activity in petunia leaf explants 2 days after infection. Similarly, using a *gusA* gene containing an intron (such that GUS activity is eliminated in Agrobacterium), Li et al. (1992) first detected GUS activity 2 days after infection of rice stem explants.

We recently detected single-stranded T-DNA in the cytoplasm of regenerating tobacco protoplasts within 30 min of cocultivation with Agrobacterium. The bacteria had been previously incubated with acetosyringone to induce *vir* gene activity and process T-DNA from the Ti plasmid (Yusibov et al., 1994). In an attempt to elucidate the kinetics of T-DNA transport to the nucleus as well as to determine which Vir proteins are necessary to effect this process, we developed a very sensitive assay to detect T-DNA transcription early after infection. The sensitivity of this assay depends on two factors. The first is the use of a "super promoter" (Ni et al., 1995) to direct high-level transcription of a *gusA* gene (containing an intron) harbored by the T-DNA. The second factor is the use of a reverse transcriptase–polymerase chain reaction (RT-PCR) assay to detect very low amounts (<10 fg) of *gusA* mRNA in the infected plant cells. This assay thus measures the very early kinetics of transcription of the T-DNA, including the molecular steps (T-DNA transfer, nuclear targeting and transport, and replication to a double-stranded T-DNA molecule) that must precede transcription. Our assay does not necessarily reflect transcription of integrated T-DNA molecules; rather, circumstantial evidence suggests that most early transcription of the T-DNA results from the transcription of nonintegrated T-DNA molecules (Janssen and Gardner, 1989).

Using this assay, we detected T-DNA transcription 18 hr after cocultivation of tobacco suspension culture cells with Agrobacterium. Transient transcription of the T-DNA absolutely depends on intact VirB, VirD1/VirD2, and VirD4 proteins. Transient transcription decreased without intact VirC and VirE2 proteins. A mutation of VirD2 that eliminates the ω domain (Shurvinton et al., 1992) resulted in an Agrobacterium strain with very weak virulence, but we showed that it still transiently directs a high level of *gusA* transcription. These results suggest that the ω domain of VirD2 is at least partially responsible for the stabilization of T-DNA transcription, perhaps by aiding T-DNA integration. The initial kinetics of *gusA* transcription in maize suspension culture cells are similar to those of tobacco cells, suggesting that T-DNA transfer, nuclear targeting, and replication to a double-stranded form occur in maize in a manner similar to that occurring in tobacco. In maize, however, transcription of the *gusA* gene is highly transient, suggesting that the block to stable transformation of maize by Agrobacterium occurs at the stage of integration of T-DNA.

RESULTS

Development of a System to Detect the Early Transcription of T-DNA

To determine the kinetics of transport of the T-DNA from the cytoplasm to the nucleus and the importance of various Vir proteins in this process, we developed a sensitive assay to detect early transcription of the T-DNA. The rationale for this line of experimentation was that the early kinetics of T-DNA expression reveal the maximal time necessary for T-DNA to translocate from the cytoplasm to the nucleus, for single-stranded T-DNA to replicate to a double-stranded form, and for transcripts to accumulate to an extent that they can be detected.

We first constructed a T-DNA binary vector, pBISN1 (Figure 1), that contains a *gusA* (*uidA*) gene under the transcriptional

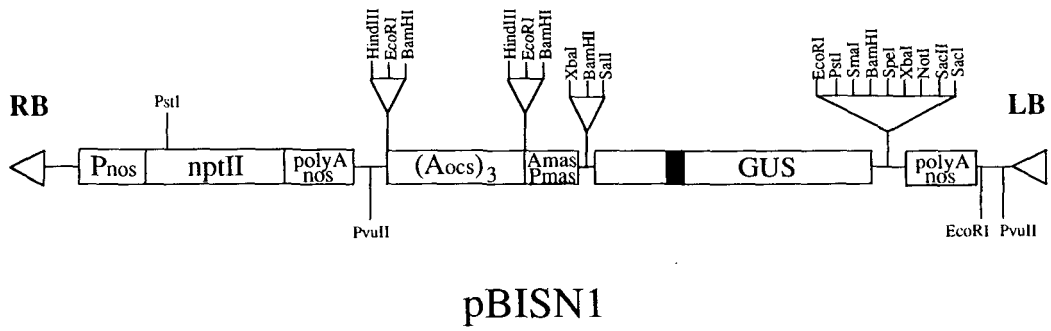

Figure 1. Map of the T-DNA Region of the Binary Vector pBISN1.

pBISN1 is based on the plasmid pBI101.2. RB, T-DNA right border; LB, T-DNA left border; Pnos, nopaline synthase promoter; nptII, neomycin phosphotransferase II coding region; polyA nos, nopaline synthase polyadenylation signal sequence; $(Aocs)_3$, trimer of the octopine synthase activating element; Amas, mannopine synthase activating element; Pmas, mannopine synthase promoter; GUS, β-glucuronidase coding region. The filled region within the *GUS* gene represents the ST-LS1 intron.

control of a super promoter (Ni et al., 1995). The *gusA* gene contains a 189-bp intron from the potato ST-LS1 gene (Vancanneyt et al., 1990). We generated PCR primers that would amplify a 732-bp region containing this intron. However, if the intron were processed from a *gusA* transcript, we would amplify (using RT-PCR) a 543-bp fragment. Because of the size difference in amplification products from the *gusA-intron* gene and the processed *gusA* transcript, we could distinguish between *gusA* mRNA and *gusA*-intron DNA from contaminating Agrobacterium cells. RT-PCR analysis of in vitro–transcribed *gusA* RNA indicated that, by using this assay, we could readily detect 10 fg of *gusA* mRNA.

We infected rapidly dividing tobacco BY-2 suspension culture cells (~20 bacterial cells per tobacco cell) with each of three Agrobacterium strains harboring pBISN1. Agrobacterium strain At793 lacks a Ti plasmid, Agrobacterium strain At789 contains the octopine-type Ti plasmid pTiA6, and Agrobacterium strain At790 contains the agropine-type supervirulent Ti plasmid pTiBo542. After cocultivation for 12 hr, the plant cells were pelleted by centrifugation, washed in plant culture medium, and resuspended in plant culture medium containing antibiotics, which were added to kill any remaining bacterial cells. We isolated total cellular RNA from infected plant cells at various times starting from the initiation of cocultivation, and we subjected the RNA to RT-PCR, using primers that would amplify the region of *gusA* mRNA flanking the (processed) intron.

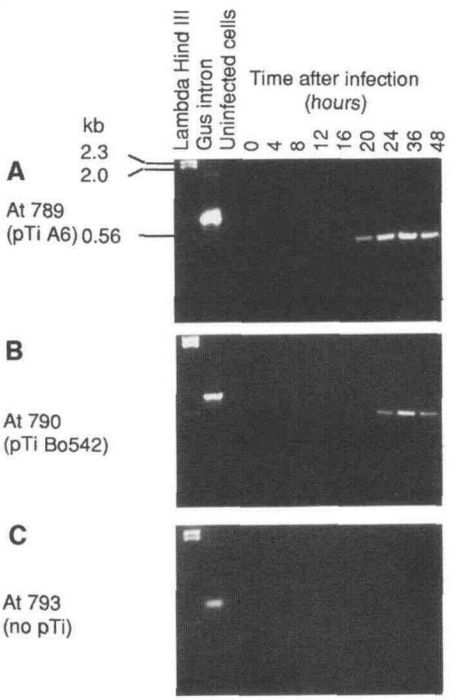

Figure 2. Kinetics of *gusA* Gene Expression (until 48 Hr) in Tobacco Suspension Culture Cells.

Tobacco cells were infected with Agrobacterium strains for various periods of time. RNA was extracted, subjected to RT-PCR, and analyzed by agarose gel electrophoresis, as described in Methods.
(A) Strain At789.
(B) Strain At790.
(C) Strain At793.
Lambda HindIII, length standards of bacteriophage λ digested with HindIII; GUS intron, PCR amplification of a 732-bp *gusA*-intron gene region.

Kinetics of Expression of T-DNA-Encoded Genes Early after Infection of Tobacco BY-2 Suspension Culture Cells

Figures 2A and 2B show that we could detect *gusA* transcripts beginning 20 to 24 hr after infection of tobacco BY-2 suspension culture cells by either Agrobacterium strain containing a Ti plasmid. The level of *gusA* mRNA increased and peaked at 36 hr, after which there was a slight decline in *gusA* mRNA. This decline was highly reproducible and occurred consistently in each of >10 independent cocultivation experiments. We could not detect *gusA* mRNA in noninfected plant cells or in plant cells from which RNA was extracted immediately after the start of cocultivation (0 hr time point). The synthesis of *gusA* mRNA in cocultivated plant cells depended on the presence of a Ti plasmid within the infecting Agrobacterium strain. Repeated analyses of RNA extracted from tobacco cells cocultivated with Agrobacterium strain At793 (lacking a Ti plasmid) never revealed the presence of *gusA* mRNA (Figure 2C). These data suggest that expression of the *gusA* gene was *vir* gene dependent. We have previously shown that the transfer of single-stranded T-DNA to the tobacco cell cytoplasm is dependent on an intact *virB* locus (Yusibov et al., 1994).

We next determined the stability of *gusA* gene expression after infection with Agrobacterium. We cocultivated tobacco BY-2 cells with various Agrobacterium strains, washed the cells 24 hr after infection, resuspended them in culture medium containing antibiotics to kill the bacteria, and isolated total cellular RNA at 24-hr intervals starting from the initiation of cocultivation. Figure 3A shows that by using Agrobacterium strain At789 (containing pTiA6), we could detect *gusA* mRNA for up to 3 days after the initiation of cocultivation. We could never detect *gusA* transcripts after 72 hr of infection. Using Agrobacterium strain At790 (containing pTiBo542), however, we routinely detected *gusA* mRNA for at least 7 days (the latest time point that we assayed; Figure 3B). Using Agrobacterium strain At793 (lacking a Ti plasmid), we again could not detect *gusA* transcripts (Figure 3C).

Cocultivation for 2 Hr Suffices to Generate Detectable Levels of T-DNA Gene Expression

To determine the minimal infection time required for synthesis of detectable levels of *gusA* mRNA, we infected tobacco BY-2 cells with Agrobacterium strain At790 (at a ratio of 100 bacterial cells per plant cell), washed the tobacco cells and

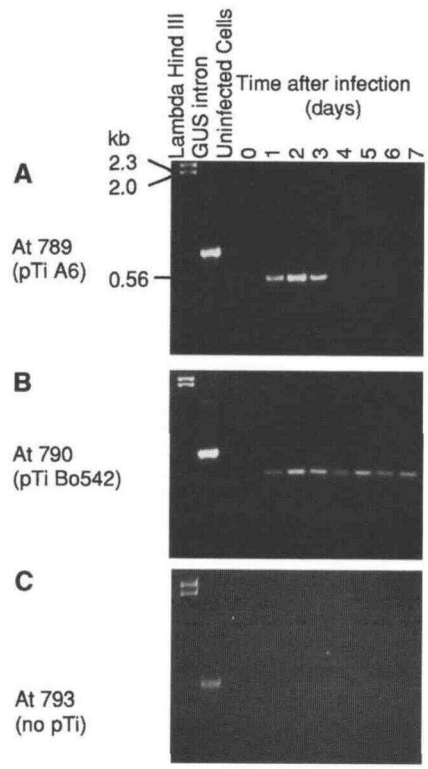

Figure 3. Kinetics of *gusA* Gene Expression (until 7 Days) in Tobacco Suspension Culture Cells.

Tobacco cells were infected with Agrobacterium strains for 24 hr, washed free of bacteria, and resuspended in culture medium plus antibiotics for various periods of time. RNA was extracted, subjected to RT-PCR, and analyzed by agarose gel electrophoresis, as described in Methods. Time points indicate the number of days after the initiation of cocultivation.

(A) Strain At789.

(B) Strain At790.

(C) Strain At793.

Lambda HindIII and GUS intron are as given in legend to Figure 2.

resuspended them in culture medium containing antibiotics at various periods of time, and extracted total cellular RNA 24 hr after the start of cocultivation. Figure 4 shows that we could detect the synthesis of *gusA* mRNA only after a minimal cocultivation period of 2 hr. We repeated this experiment using higher inoculation ratios of bacterial cells to plant cells (ranging up to 2000:1), but we were never able to detect the presence of *gusA* transcripts with a cocultivation period of <2 hr (data not shown). At higher ratios of bacterial to plant cells used for inoculations, the bacteria rapidly overgrew and killed the plant cells. The 2-hr minimal cocultivation period necessary to detect *gusA* transcripts correlates well with the time (2 hr) of maximal single-stranded T-DNA accumulation in the tobacco cell cytoplasm after the initiation of cocultivation (Yusibov et al., 1994).

Quantitation of *gusA* Transcripts in Infected Tobacco Cells

To determine the quantity of *gusA* mRNA molecules in cocultivated plant cells, we first determined the percentage of tobacco cells infected by Agrobacterium. We assayed GUS activity by staining the infected tobacco cells with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) at various times after infection. Because infected plant material consisted of small clusters of cells rather than individual cells and because the tobacco cells continued to divide during this experiment, we estimated the percentage of infected cells by counting the percentage of cell clusters, rather than individual cells, that stained blue. Table 1 shows that we first detected GUS activity 2.5 days after infection. The percentage of stained cells increased thereafter and then stabilized by day 5 after infection. The fact that we could detect *gusA* mRNA by using RT-PCR after 18 to 24 hr of infection but could detect GUS activity only starting 2.5 days after infection most likely reflects the relative sensitivity of these two assays. Infection of a particular batch of tobacco BY-2 cells with Agrobacterium strain At790 (containing the supervirulent Ti plasmid pTiBo542) consistently resulted in an ~10-fold higher percentage of infected cells than did infection of the same batch of cells with Agrobacterium strain At789 (containing the octopine-type Ti plasmid pTiA6). We used the data in Table 1 to calculate the quantity of *gusA* mRNA as determined in Figure 5B. However, the percentage of infected cells differed from experiment to experiment. Cocultivation with Agrobac-

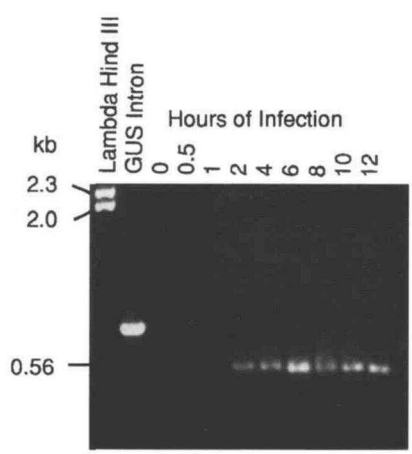

Figure 4. Cocultivation Time Requirement for *gusA* Gene Expression in Tobacco Suspension Culture Cells.

Tobacco cells were infected with Agrobacterium strain At790 for various periods of time, after which the plant cells were washed and resuspended in medium containing antibiotics to kill the bacteria. The tobacco cells were harvested 24 hr after the start of cocultivation; the RNA was then extracted, subjected to RT-PCR, and analyzed by agarose gel electrophoresis, as described in Methods. Lambda HindIII and GUS intron are as given in legend to Figure 2.

Table 1. Percentage of Tobacco Cells Stained Blue by Using X-gluc

Agrobacterium Strain	Ti Plasmid	Days after the Initiation of Cocultivation[a]			
		2.5	3	4	5
At793	No pTi	0.00	0.00	0.00	0.00
At789	pTiA6	0.00	0.1 ± 0.08	0.5 ± 0.07	1.0 ± 0.14
At790	pTiBo542	0.1 ± 0.04	1.8 ± 0.28	6.6 ± 0.43	7.3 ± 0.92
At805	pTiA6 *virB1*⁻	0.00	0.00	0.00	0.00
At806	pTiA6 *virD1*⁻	0.00	0.00	0.00	0.00
At807	pTiA6 *virE2*⁻	0.00	0.00	0.00	0.00
At808	pTiA6 *virD2*⁻ (nonpolar)	0.00	0.00	0.00	0.3 ± 0.07
At871	pTiA6 *virC1*⁻	0.00	0.00	<0.01[b]	0.00
At819	pTiA6 *virD4*⁻	0.00	0.00	0.00	0.00
At822	pTiA6	0.12	0.13	0.23	0.23
At829	pTiA6 *virD2*⁻ (ω substitution)	0.00	0.00	<0.01b	0.00

[a] Data represent the mean of three or more independent experiments. A minimum of 3000 cell clusters were scored for each time point.
[b] Occasional blue-staining cells observed.

terium strain At789 ultimately resulted in infection of 0.1 to 1.0% of the cells, whereas cocultivation with Agrobacterium strain At790 ultimately resulted in infection of 1.0 to 7.3% of the cells.

We next determined the quantity of *gusA* mRNA present per microgram of total tobacco cellular RNA. To do this, we first established the sensitivity of our RT-PCR reaction. Figure 5A shows that when we mixed various amounts of in vitro–transcribed *gusA*-intron mRNA with 1 μg of total tobacco RNA, we could detect 10 fg of *gusA* RNA.

We next mixed various quantities of in vitro–transcribed (standard) *gusA*-intron RNA with 1 μg of RNA extracted from tobacco cells 36 hr after the start of cocultivation and subjected the mixture to RT-PCR. By conducting a "competition" between the processed sample of *gusA* mRNA and the unprocessed "standard" *gusA* mRNA, we could determine the concentration at which amplification of each mRNA yielded approximately the same intensity signal. Comparison of the intensity of the 732-bp (standard *gusA*-intron) cDNA signal with the 543-bp processed *gusA* cDNA signal indicated that, unexpectedly, populations of tobacco cells cocultivated with either Agrobacterium strain At789 or At790 contained approximately the same amount of *gusA* mRNA. At 36 hr after infection with strain At789, ~0.05 pg of *gusA* mRNA was present per microgram of total cellular RNA from all cells. For tobacco cells infected with strain At790, ~0.01 pg of *gusA* mRNA was present per microgram of total cellular RNA from all cells (Figure 5B). We repeatedly obtained this result despite the ~10-fold difference in the percentage of infected cells, as determined by X-gluc staining, with use of the two Agrobacterium strains (Table 1).

Considering the percentage of cocultivated cells that ultimately produced GUS activity (~0.1 to 1.0% after infection by strain At789 and ~1.0 to 7.3% after infection by strain At790; Table 1) and assuming that ~1% of the total tobacco cellular RNA is poly(A)$^+$ mRNA, we calculated that ~0.02% of the

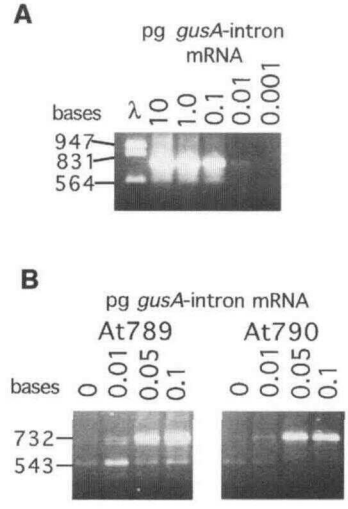

Figure 5. Quantitation of *gusA* mRNA in Infected Tobacco Cells.

(A) Determination of the sensitivity of the RT-PCR reaction. Various amounts of in vitro–transcribed *gusA*-intron mRNA were mixed with 1 μg of total tobacco RNA. The reaction mixture was subjected to RT-PCR, and the PCR products were analyzed by agarose gel electrophoresis, as described in Methods.

(B) Quantitation of *gusA* mRNA present in tobacco cells 36 hr after the initiation of cocultivation with Agrobacterium strain At789 (an octopine-type strain) or At790 (an agropine-type strain). Various amounts of in vitro–transcribed *gusA*-intron mRNA were mixed with 1 μg of total RNA from infected tobacco, and the mixture was subjected to RT-PCR, as described in Methods. The band at 732 bases represents the PCR amplification product of the in vitro–transcribed *gusA*-intron mRNA, whereas the 543-base fragment represents the amplification product of the *in vivo*–processed *gusA* mRNA. λ, HindIII restriction endonuclease fragments of bacteriophage λ.

poly(A)$^+$ mRNA was represented by *gusA* mRNA when tobacco cells were cocultivated with Agrobacterium strain At790. When Agrobacterium strain At789 was used, ~1.0% of the poly(A)$^+$ mRNA was represented by *gusA* transcripts. We made these calculations using RT-PCR data taken from the 36-hr time point (the time of maximal expression of *gusA* mRNA) and X-gluc staining data from day 5. In making these calculations, we assumed that the tobacco cells were maximally transformed by 36 hr (because the bacteria had been killed by that time), but we could not determine the maximal percentage of transformed cells until day 5 because of the insensitivity of the X-gluc staining assay relative to the RT-PCR assay.

Dependence of Early T-DNA Transcription on *vir* Gene Function

To determine the importance of different vir proteins in the early stages of T-DNA transfer, transcription, and perhaps integration, we introduced pBISN1 or pBISN2 into Agrobacterium containing mutations in various *vir* genes. Initially, we investigated mutant bacterial strains harboring Tn*3*-HoHo1 insertions (Stachel and Nester, 1986) in *virB1* (mx243; At805), *virC1* (mx365; At871), *virD1* (mx311; At806), *virD4* (mx367; At819), *virE2* (mx341; At807), and a nonpolar insertion in the 3′ end of *virD2* (mx304; At808). We incubated each mutant strain separately with tobacco BY-2 suspension culture cells, washed the plant cells and killed the bacteria with antibiotics after cocultivation for 12 hr, extracted tobacco RNA at 18, 24, 36, and 48 hr after the initiation of cocultivation, and assayed for the presence of *gusA* mRNA by RT-PCR.

Figure 6A shows that Agrobacterium At789 containing wild-type *vir* genes directed the synthesis of *gusA* mRNA in tobacco cells to a maximal extent at 36 hr, similar to the result shown in Figure 2A. Figure 6B shows that the Agrobacterium At805 mutant in *virB* could not direct the synthesis of *gusA* mRNA in infected tobacco cells. We have previously shown that this same strain could not transfer T-DNA to the cytoplasm of tobacco cells (Yusibov et al., 1994), although wild-type levels of T strands accumulated in protoplast-induced (Veluthambi et al., 1988) or acetosyringone-induced (Stachel et al., 1987) bacterial cells. Similarly, Agrobacterium strain At819, containing a mutant *virD4* gene, could not effect *gusA* transcription in cocultivated tobacco cells (Figure 6H). VirD4 is a bacterial periplasmic membrane-localized protein (Okamoto et al., 1991). Agrobacterium or *Escherichia coli* strains containing mutant *virD4* genes can process T-DNA and accumulate T strands (Jayaswal et al., 1987; Stachel et al., 1987). Agrobacterium cells mutant in *virD4* may not, however, be able to export T strands through the VirB protein export apparatus. Agrobacterium strain At806, containing a polar insertion in *virD1*, could not direct *gusA* mRNA synthesis (Figure 6E). An Agrobacterium strain containing this same *virD1* mutation could not process T-DNA or accumulate T strands (Stachel et al., 1987; Veluthambi et al., 1988).

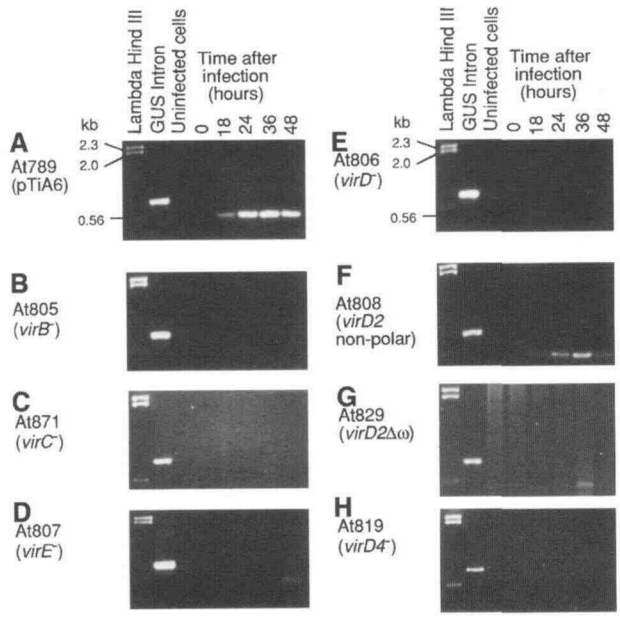

Figure 6. Effect of *vir* Gene Mutations on the Kinetics of *gusA* Gene Expression (until 48 Hr) in Tobacco Suspension Culture Cells.

Tobacco cells were infected with Agrobacterium strains for various periods of time. RNA was extracted, subjected to RT-PCR, and analyzed by agarose gel electrophoresis, as described in Methods.

(A) Strain At789.
(B) Strain At805.
(C) Strain At871.
(D) Strain At807.
(E) Strain At806.
(F) Strain At808.
(G) Strain At829.
(H) Strain At819.

Lambda HindIII and GUS intron are as given in legend to Figure 2.

Cocultivation of tobacco cells with Agrobacterium strain At807 (mutant in *virE*) resulted in only a low level of *gusA* mRNA accumulation late (48 hr) in the course of infection (Figure 6D). Stachel and Nester (1986) have shown that similar mutations in *virE* greatly attenuate bacterial virulence, despite the fact that normal levels of T strands accumulate in induced cells (Stachel et al., 1987; Veluthambi et al., 1988). Thus, the presence of a VirE2 single-stranded DNA binding protein is not necessary to protect the T strand within the bacterium. The low level of *gusA* transcripts that accumulated late after infection reflects the low steady state level of single-stranded T-DNA that accumulates in the cytoplasm of infected tobacco cells (Yusibov et al., 1994).

Mutations in *virC* severely attenuate the virulence of Agrobacterium on most plant species, including tobacco (Stachel and Nester, 1986), although nearly wild-type levels of T strands accumulate in induced bacterial cells (Stachel et al., 1987; Veluthambi et al., 1988). Agrobacterium strain At871, mutant

in *virC*, could direct only a very low level of transient *gusA* mRNA accumulation in infected tobacco cells (Figure 6C).

Agrobacterium strains containing the nonpolar insertion mx304 in the 3′ end of *virD2* are avirulent (Stachel and Nester, 1986; Koukolikova-Nicola et al., 1993), although they accumulate T strands after induction by acetosyringone (Stachel and Nester, 1986). The Tn*3*-HoHo1 insertion is separated by 73 amino acids from the C terminus of VirD2 and translationally fuses the β-galactosidase protein to the C terminus of VirD2 (Koukolikova-Nicola et al., 1993). The resulting altered VirD2 protein lacks the C-terminal NLS (Howard et al., 1992; Tinland et al., 1992) and the ω region that is important for efficient tumorigenesis in potato (Shurvinton et al., 1992) and tobacco (S. Gelvin, unpublished data). Therefore, we were surprised to detect relatively high levels of *gusA* transcripts in tobacco cells infected with Agrobacterium strain At808 harboring this *virD2* mutation (Figure 6F).

Quantitative RT-PCR indicated that the level of *gusA* transcript resulting from infection of tobacco cells by this mutant Agrobacterium strain was 20 to 30% of that found in tobacco cells cocultivated with strain At789 containing a wild-type *virD2* gene (data not shown). The high level of *gusA* mRNA in these cells was considerably more transient than that resulting from infection by the wild-type bacterium. Arabidopsis roots infected by a similar Agrobacterium strain harboring the same nonpolar *virD2* mutation showed ⩾50% of the GUS activity present in roots infected by a wild-type Agrobacterium strain (J. Nam and S.B. Gelvin, unpublished data). These data indicate that deletion of the C-terminal NLS and/or the ω region of VirD2 results in high-level transient expression of T-DNA–encoded genes. The high level of transient *gusA* gene expression after infection of tobacco cells by strain At808 suggests that, despite the lack of a C-terminal NLS in the VirD2 protein encoded by this strain, the T-DNA is efficiently directed to the plant nucleus.

The translational fusion of β-galactosidase protein to the C terminus of VirD2 in Agrobacterium strain At808 could result in a protein with altered properties. Therefore, we repeated the tobacco cell infections, using strain At829. This strain encodes a mutated VirD2 protein containing two serine residues in place of four of the five amino acids in the ω region (Shurvinton et al., 1992); therefore, it lacks the ω region yet still retains the NLS. Infection of potato tuber discs (Shurvinton et al., 1992) or tobacco leaf sections (S.B. Gelvin, unpublished data) with a similar Agrobacterium strain harboring this same *virD2* mutation resulted in 3.3 and 2.5%, respectively, of the number of tumors present in infection with a strain harboring a wild-type *virD2* gene. Figure 6G shows that strain At829 directed a high level of transient transcription of the *gusA* gene. Quantitative RT-PCR indicated that similar to Agrobacterium strain At808, *gusA* transcripts accumulated up to 20 to 30% of the level of wild-type strain At822 in infected tobacco cells (data not shown). These data indicate that the ω region of VirD2 is not required for T-DNA transport to the plant cytoplasm or nucleus, for replication to a double-stranded form, or for transcription. The data suggest, however, that this region of VirD2 may be involved in the stabilization of T-DNA transcription, perhaps by mediating integration of T-DNA into the plant genome.

Transient Expression of *gusA* mRNA in Maize Suspension Culture Cells

Ritchie et al. (1993) and Shen et al. (1993) have shown that Agrobacterium can transfer T-DNA to maize tissue explants and that infected tissue can express GUS activity. However, these authors did not extensively investigate the stability of *GUS* expression. Considering the high stability of GUS enzymatic activity in many plant tissues (Jefferson et al., 1987), we thought it particularly important to investigate the stability of expression of *gusA* mRNA in infected maize cells. We therefore cocultivated maize Black Mexican Sweet (BMS) suspension culture cells with Agrobacterium strain At789 (harboring pTiA6) or At790 (harboring pTiBo542) that had been previously induced with acetosyringone. Figure 7 shows that by using RT-PCR, we could detect *gusA* transcripts within infected maize cells 24 hr after infection when either strain was used. The appearance of *gusA* mRNA was, however, very transient. By 36 hr after the initiation of cocultivation, the level of *gusA* mRNA had decreased greatly, and it was never detected at 48 hr. We detected the same kinetics of transcript accumulation and disappearance in three independent cocultivation experiments. Thus, the kinetics of initial appearance of *gusA* mRNA in maize approximated that of tobacco. In maize, however, the presence of *gusA* mRNA was highly transient, suggesting that in this monocot species, T-DNA integration and/or stable transcription was defective.

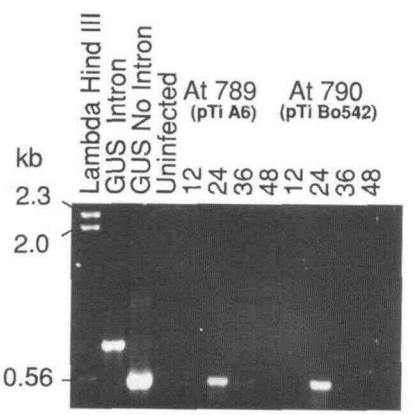

Figure 7. Kinetics of *gusA* Gene Expression (until 48 Hr) in Maize Suspension Culture Cells.

Maize cells were infected with Agrobacterium strains At789 or At790 for various periods of time (hours). RNA was extracted, subjected to RT-PCR, and analyzed by agarose gel electrophoresis, as described in Methods. Lambda HindIII, length standards of bacteriophage λ digested with HindIII; GUS intron, PCR amplification of a 732-bp *gusA*-intron gene region; GUS no intron, PCR amplification of a 543-bp *gusA* gene region (lacking an intron).

DISCUSSION

A New System to Investigate the Early Events of T-DNA Expression in Plants

We developed an experimental system to investigate the expression of T-DNA–encoded genes in plant cells soon after infection by Agrobacterium. Because this system uses acetosyringone-induced Agrobacterium cells cocultivated with plant suspension culture cells rather than regenerating plant protoplasts, it is easier to use than many previously described cocultivation procedures (Fraley et al., 1983; Horsch et al., 1985). Infection of rapidly dividing tobacco cells by Agrobacterium was reported by An (1985). In these experiments, he noted that maximal induction of bacterial *vir* genes by the plant cells required 2 to 3 days. We induced Agrobacterium *vir* genes with acetosyringone for 14 to 18 hr before cocultivation. Therefore, T-DNA processing and the establishment of the VirB-mediated T-DNA transport machinery had already taken place. We have shown that by using these infection conditions and regenerating tobacco protoplasts, we could detect T-DNA transfer to the plant cytoplasm within 30 min of infection (Yusibov et al., 1994). Therefore, we believe that our infection protocol results in a rapid and relatively synchronous "burst" of T-DNA transfer to plant cells and is therefore useful for studying the initial kinetics of T-DNA transfer processes.

Several other aspects of our system make it especially amenable for investigating the early molecular events of T-DNA expression in plant cells. The use of RT-PCR allowed us to detect very small quantities (10 fg) of *gusA* mRNA. By using PCR primers that span an intron-encoding sequence within the *gusA* gene, we could easily distinguish the amplified product derived from processed *gusA* mRNA from that of the *gusA* gene that may have been present due to contaminating Agrobacterium cells. However, we rarely detected the larger amplified product, most likely because of the DNase I treatment of the tobacco nucleic acids before PCR amplification. Finally, the use of a strong, constitutive super promoter (Ni et al., 1995) to direct expression of the *gusA* gene provided an additional level of sensitivity essential for conducting these experiments. Repeated attempts to detect *gusA* transcripts within the first 2 days after the start of cocultivation failed when we used the cauliflower mosaic virus 35S promoter to direct expression of the *gusA* gene.

Kinetics and Stability of T-DNA Transfer and Expression in Tobacco Cells

Using these protocols for infection and analysis, we could detect *gusA* gene expression in tobacco cells as early as 18 hr after infection (Figure 6). Because we have previously shown that T-DNA transfer to the plant cytoplasm is very rapid with these procedures, this interval represents the time necessary for transport of the T-DNA to the nucleus, conversion to a double-stranded molecule, and transcription of the *gusA* gene to a level detectable by RT-PCR. T strands are generated from the right to the left T-DNA border (Stachel et al., 1986; Veluthambi et al., 1988). Thus, because of the orientation of the *gusA* gene in pBISN1 relative to the right border, the coding strand of the *gusA* gene is transferred to the plant as the T strand. Therefore, the template for transcription of *gusA* will be present only after conversion of the single-stranded T strand to a double-stranded form. In addition, the nuclear-localized RNA polymerase II that transcribes T-DNA genes (Willmitzer et al., 1981) requires a double-stranded DNA template. Transcription of such a double-stranded DNA molecule does not require the integration of T-DNA into the plant genome.

The earliest previous detection of T-DNA–encoded activity occurred 2 days after infection (nopaline synthase activity [Fraley et al., 1984] or GUS activity [Janssen and Gardner, 1989; Castle and Morris, 1990; Li et al., 1992]). In our experiments, the levels of *gusA* mRNA increased until 36 hr after infection and then declined (Figures 2, 3, and 6). Janssen and Gardner (1989) described a similar decline in the expression of GUS activity after 3 days of infection of petunia leaf sections. These authors attributed this pattern of expression to the transient presence and expression of nonintegrated copies of the T-DNA in the nucleus. We interpret our data similarly. Although it is formally possible that the decline in T-DNA expression resulted from a repression of *gusA* transcription after integration of the T-DNA into the plant genome, we think that the large and rapid decrease in *gusA* mRNA levels that we observed was not likely to occur in such a manner.

Tobacco cells infected with Agrobacterium strain At789 (harboring the octopine-type Ti plasmid pTiA6) contained detectable amounts of *gusA* mRNA for only 3 days after infection, whereas tobacco cells infected with Agrobacterium strain At790 (harboring the agropine-type supervirulent Ti plasmid pTiBo542) expressed detectable levels of *gusA* transcripts for ≥7 days. We do not interpret the transient nature of detectable *gusA* mRNA accumulation to indicate that tobacco cells infected with strain At789 only briefly contain T-DNA; indeed, this strain is virulent. We speculate, however, that most of the initial *gusA* transcription results from nonintegrated copies of T-DNA and that use of strain At789 caused the extent of T-DNA integration and expression to fall below our level of detection. We further speculate that the continuous expression of *gusA* mRNA in tobacco cells infected with strain At790 reflects a higher level of *gusA* transcription after integration of T-DNA. Increased integration might be attributed to more transfer of T-DNA to the plant, a higher efficiency of T-DNA integration (perhaps catalyzed by the supervirulent VirD2 or VirE2 proteins), or both. We are currently developing quantitative T-DNA integration assays to investigate these events.

To measure the amount of *gusA* mRNA present in infected tobacco cells, we added various known amounts of in vitro–transcribed *gusA* mRNA (containing an intron) to 1 μg of total RNA extracted from cocultivated plant cells and sub-

jected the mixture to RT-PCR. Comparison of the PCR signal intensity of the two amplification products indicated the amount of *gusA* mRNA among the total population of tobacco RNA. Although the quantity of *gusA* mRNA per microgram of total RNA was higher after infection of tobacco cells with Agrobacterium strain At790 than with strain At789, fewer cells stained blue when the octopine-type strain was used. Thus, it appears that strain At789 directed a higher level of *gusA* transcript accumulation (per infected cell) than did the supervirulent strain At790. We suggest that this paradox may derive from an artifact of our quantitation procedure and may best be explained by assuming that X-gluc staining may underestimate the percentage of cells infected by failing to detect those that did not express GUS activity long enough to result in blue staining. For example, we could never detect blue staining of maize cells after transformation by Agrobacterium, although we could detect transient *gusA* gene expression by using RT-PCR (Figure 7). Thus, we may not be able to detect GUS activity, as determined by blue staining of cells with X-gluc, until *gusA* mRNA accumulates to a particular threshold level. Similarly, we may not be able to detect GUS activity if *gusA* mRNA is not stable for a long enough time to become translated to a threshold level of GUS protein.

Effects of *vir* Gene Mutations on the Early Events of T-DNA Expression

By using mutant Agrobacterium strains, we established the importance of various *vir* gene products in the expression of *gusA* mRNA. Mutations in *virB* and *virD4* result in loss of virulence (Stachel and Nester, 1986), despite the fact that T-DNA processing and T strand accumulation in the bacteria are not impaired (Stachel et al., 1987; Veluthambi et al., 1988). VirB proteins presumably make up the channels through which T-DNA and some Vir proteins exit from the bacteria (Thompson et al., 1988; Ward et al., 1988; Kuldau et al., 1990), and Agrobacterium strains mutant in *virB* cannot transfer T-DNA to the cytoplasm of regenerating tobacco protoplasts (Yusibov et al., 1994). The VirD4 protein associates with the bacterial inner membrane and may play a role in T-DNA export from the bacterium (Okamoto et al., 1991). Therefore, we expected that Agrobacterium strains mutant in these genes would not direct the expression of *gusA* mRNA in cocultivated plant cells, and they did not (Figure 6). An Agrobacterium strain containing a polar mutation in *virD1* also did not induce *gusA* transcription in tobacco cells. A similar strain harboring the same *virD1* mutation was unable to process T-DNA and generate T strands (Stachel et al., 1987).

Agrobacterium strains mutant in *virC* and *virE* demonstrate a highly attenuated virulence phenotype (Stachel and Nester, 1986), although they accumulate wild-type levels of T strands (Stachel et al., 1987; Veluthambi et al., 1988). They must therefore be defective in processes involved in virulence subsequent to T-DNA processing. We have shown (Figure 6) that these mutants direct only a very low level of *gusA* transcription, corresponding to the low level of tumorigenesis. We have previously demonstrated that an Agrobacterium *virE* mutant can transfer T strands to the cytoplasm of regenerating tobacco protoplasts (Yusibov et al., 1994). However, the steady state level of T-DNA that accumulates in the plant cytoplasm was ~20% of that found when the protoplasts were infected by a wild-type strain. The VirE2 protein binds to single-stranded DNA molecules (Gietl et al., 1987; Christie et al., 1988; Citovsky et al., 1988; Sen et al., 1989) and may therefore protect the T strand in the plant cell. We speculate that the low level of delayed *gusA* transcription that we detected subsequent to infection of tobacco cells with an Agrobacterium *virE* mutant resulted from the transport of fewer intact T strands to the nucleus and the subsequent lower initial levels of T-DNA transcription. The likely importance of the VirE2 protein in the plant cell, but not in the bacterial cell, has been suggested by "extracellular" complementation of Agrobacterium *virE2* mutants by coinoculation of plants with an Agrobacterium strain harboring an intact *virE* locus but no T-DNA (Otten et al., 1984). The restoration of tumorigenesis by infection of transgenic tobacco plants that synthesize the VirE2 protein with an avirulent Agrobacterium *virE2* mutant (Citovsky et al., 1992) further suggests an important function for VirE2 protein in the plant cell.

Infection of tobacco BY-2 cells with an Agrobacterium strain containing the transposon Tn3-HoHo1 in *virD2* (mx304) resulted in the transient accumulation of *gusA* mRNA to levels ~20 to 30% of that found after infection by wild-type strains (Figure 6). A similar strain harboring this same transposon insertion either is avirulent (Stachel and Nester, 1986) or shows extremely attenuated virulence ($\leq 10^{-6}$ to 10^{-5}) on host plants (Koukolikova-Nicola et al., 1993). Koukolikova-Nicola et al. (1993) also claimed that this strain shows low transient GUS expression and can incite disease symptoms on turnip plants at only 0.1 to 10% of the level of wild-type Agrobacterium strains after agroinfection with cauliflower mosaic virus. The transposon insertion in Agrobacterium mx304 results in deletion of the last 73 amino acids of VirD2 (Koukolikova-Nicola et al., 1993), including the C-terminal NLS and the conserved ω domain (Shurvinton et al., 1992). This mutation also translationally fuses β-galactosidase to the C terminus of the VirD2 protein.

Our finding that infection of plants by this avirulent strain still resulted in a relatively high (20 to 30% of that of the wild type) level of *gusA* transcript accumulation suggests that deletion of the C-terminal NLS and ω domains does not markedly inhibit nuclear transport of the T strand or its conversion to a double-stranded form and apparently contradicts the conclusions of Koukolikova-Nicola et al. (1993). We propose that deletion of the C-terminal NLS of the VirD2 protein can be partially compensated for by NLS sequences within VirE2 protein molecules that presumably coat T strands in the plant cell (Citovsky et al., 1992). In support of this conclusion, Shurvinton et al. (1992) showed that a precise deletion of both of the four amino acid domains constituting the C-terminal NLS results

in only a 40% reduction of virulence, as measured by a quantitative potato tuber disc assay.

In addition, we have shown that such a precise deletion of the C-terminal NLS results in only a slight decrease in transient GUS activity in infected Arabidopsis roots (J. Nam and S.B. Gelvin, unpublished data) and a similar slight decrease in the transient accumulation of *gusA* mRNA in infected tobacco BY-2 cells (X.-b. Deng and S.B. Gelvin, unpublished data). Furthermore, our data demonstrate that the ω domain of the VirD2 protein is not essential for T-DNA transfer to the plant or for T-DNA nuclear targeting.

In contrast to our conclusions that the ω domain of VirD2 is not important for nuclear transport of T strands, our data implicate this domain in T-DNA integration into the plant genome. Replacement of the four ω domain amino acids DDGR by two serine residues reduced virulence to 3.3% of the wild-type strain on potato tuber discs (Shurvinton et al., 1992) and to 2.5% on tobacco leaf explants (S.B. Gelvin, unpublished data). However, the accumulation of *gusA* mRNA in infected tobacco BY-2 cells when using this ω mutation was still 20 to 30% of that of the wild type (Figure 6). This expression was more transient than expression using an Agrobacterium strain containing a wild-type *virD2* gene. These results suggest that *gusA* transcription was not stabilized in the tobacco cells, perhaps because the T-DNA did not integrate into the tobacco genome. We are currently investigating the importance of the ω domain of the VirD2 protein in both the extent and precision of T-DNA integration into plant DNA.

T-DNA Is Rapidly Transferred to but Transiently Expressed in Maize Cells

Many monocot species, especially the agronomically important cereal grains, are highly recalcitrant to transformation by Agrobacterium. The reasons for this recalcitrance are not known, although agroinoculation of maize with maize streak virus (Grimsley et al., 1987) and the transient expression of GUS activity in Agrobacterium-infected maize tissues (Ritchie et al., 1993; Shen et al., 1993) indicate that T-DNA can be transferred from Agrobacterium into maize cells.

We showed that infection of rapidly dividing maize BMS cells by two Agrobacterium strains results in the highly transient expression of *gusA* transcripts (Figure 7). The initial kinetics of appearance of these transcripts in maize (24 hr) and in tobacco (18 to 20 hr) are approximately equal. Thus, there does not appear to be any major difference in T-DNA transfer, nuclear targeting, conversion to a double-stranded DNA form, or transcription between tobacco BY-2 cells and maize BMS cells. However, transcription of the *gusA* gene in tobacco cells continued for 3 days to at least 7 days (depending on the Agrobacterium strain used for infection), whereas transcripts disappeared from maize cells by 36 hr after infection. These data indicate that the transcription of transgenes introduced by Agrobacterium-mediated infection of maize is highly transient and suggest that the block to maize transformation may be at the level of T-DNA integration into the genome. We are currently conducting experiments to test this hypothesis directly.

METHODS

Bacterial Strains and Growth Conditions

We grew *Escherichia coli* strains at 37°C on Luria-Bertani medium (Maniatis et al., 1982) and *Agrobacterium tumefaciens* strains at 30°C on AB-sucrose minimal medium (Lichtenstein and Draper, 1986) containing the appropriate antibiotics. Antibiotic concentrations (μg/mL) are as follows: ampicillin, 100; kanamycin, 20 for *E. coli*; carbenicillin, 100; kanamycin, 100; spectinomycin, 100; rifampicin, 10 for Agrobacterium. Table 2 lists the Agrobacterium strains used in this study and their relevant characteristics. We conducted these experiments under P1 containment conditions as specified by the National Institutes of Health recombinant DNA guidelines.

Construction of pBISN1 and Its Derivatives

To construct the transferred (T)-DNA binary vector pBISN1, we first cloned an EcoRI-SalI fragment of pCNL65 (Liu et al., 1992), containing a β-glucuronidase *gusA* gene with the ST-LS1 second intron (Vancanneyt et al., 1990), into pBluescript SK+ (Stratagene). We digested this plasmid with XhoI (upstream of the *gusA* gene), filled in the overhanging ends, using the Klenow fragment of DNA polymerase I and nucleotide triphosphates, and released the *gusA*-intron gene using SacI. We removed the *gusA* gene (lacking an intron) from pE1120 (Ni et al., 1995) by using SmaI and SacI and replaced this *gusA* gene fragment with the *gusA*-intron gene fragment described above. The final plasmid, pBISN1, contains T-DNA border repeat sequences, a nopaline synthase-neomycin phosphotransferase II gene for selection of kanamycin-resistant transgenic plants, and a *gusA*-intron gene under the regulation of the "super promoter" from pE1120. pBISN1 is based on the IncP replicon RK2. To place the entire T-DNA region of pBISN1 into an IncW replicon, we digested pBISN1 with BglII (which cuts just outside the left and right T-DNA borders) and cloned the T-DNA-containing fragment into the large BamHI fragment of a derivative of pUCD2 (Close et al., 1984) that had been digested with PstI and treated with T4 DNA polymerase to disrupt the β-lactamase gene. We designated the resulting plasmid pBISN2.

We mobilized pBISN1 or pBISN2 into Agrobacterium strains, using a triparental mating procedure (Figurski and Helinski, 1979) and the mobilizing plasmid pRK2013 (Ditta et al., 1980). We selected transconjugants on AB-sucrose minimal medium containing rifampicin and kanamycin (pBISN1) or rifampicin and spectinomycin (pBISN2).

Growth and Infection of Plant Cells and Determination of GUS Activity

We propagated *Nicotiana tabacum* BY-2 cells in Murashige and Skoog medium (Gibco BRL) containing 3% sucrose, 1 μg/mL thiamine, 0.2 μg/mL 2,4-D, and 370 μg/mL KH_2PO_4. We propagated *Zea mays* Black

Table 2. Agrobacterium Strains Used in This Study

Strain	Description	Antibiotic Resistance[a]	References
At2 (A136)[b]	C58 lacking a Ti plasmid	Rif	Montoya et al. (1978)
At6 (A348)[b]	C58 containing pTiA6	Rif	Knauf and Nester (1982)
At77 (A281)[b]	C58 containing pTiBo542	Rif	Sciaky et al. (1978)
At793	pBISN1 in A136	Rif, Kan	This study
At789	pBISN1 in A348	Rif, Kan	This study
At790	pBISN1 in A281	Rif, Kan	This study
At44	A348 (mx243); Tn*3*-HoHo1 in *virB1*	Rif, Cb	Stachel and Nester (1986)
At50	A348 (mx365); Tn*3*-HoHo1 in *virC1*	Rif, Cb	Stachel and Nester (1986)
At51	A348 (mx311); Tn*3*-HoHo1 in *virD1*	Rif, Cb	Stachel and Nester (1986)
At810	A348 (mx367); Tn*3*-HoHo1 in *virD4*	Rif, Cb	Stachel and Nester (1986)
At53	A348 (mx341); Tn*3*-HoHo1 in *virE2*	Rif, Cb	Stachel and Nester (1986)
At219	A348 (mx304); Tn*3*-HoHo1 in *virD2*	Rif, Cb	Stachel and Nester (1986)
At805	pBISN1 in At44	Rif, Cb, Kan	This study
At871	pBISN1 in At50	Rif, Cb, Kan	This study
At806	pBISN1 in At51	Rif, Cb, Kan	This study
At819	pBISN1 in At810	Rif, Cb, Kan	This study
At807	pBISN1 in At53	Rif, Cb, Kan	This study
At808	pBISN1 in At219	Rif, Cb, Kan	This study
At747 [WR1753][c]	Wild-type *virD2* in WR1715[d]	Rif, Cb, Kan	Shurvinton et al. (1992)
At746 [WR1826][c]	*virD2* ω substitution in WR1715[d]	Rif, Cb, Kan	Shurvinton et al. (1992)
At822	pBISN2 in At747	Rif, Cb, Kan, Spc	This study
At829	pBISN2 in At746	Rif, Cb, Kan, Spc	This study

[a] Rif, rifampicin; Kan, kanamycin; Cb, carbenicillin; Spc, spectinomycin.
[b] Numbers within parentheses are Nester laboratory strain numbers.
[c] Numbers within brackets are Ream laboratory strain numbers.
[d] WR1715 is an octopine-type Agrobacterium strain harboring a large deletion in *VirD2* (Shurvinton et al., 1992).

Mexican Sweet (BMS) cells in Murashige and Skoog medium containing 2% sucrose, 2 μg/mL 2,4-D, 0.2 mg/mL myoinositol, 0.13 mg/mL L-asparagine, 0.13 μg/mL nicotinic acid, and 0.25 μg/mL each of thiamine, pyridoxine, and pantothenic acid. The cultures were shaken at 140 rpm at 25°C in continuous light.

To infect plant cells, we first induced virulence (*vir*) gene activity in Agrobacterium with acetosyringone. We grew Agrobacterium cells to a density of 2×10^9 cells per mL (A = 100, using a Klett-Summerson spectrophotometer, red filter) in AB–sucrose medium. We centrifuged the cells at 10,000*g*, suspended them at a concentration of 1×10^9 cells per mL (A = 50) in induction medium (AB salts, 0.5% glucose, 2 mM sodium phosphate, 50 mM Mes, pH 5.6, 50 μM acetosyringone), and incubated them with gentle shaking at 25°C for 14 to 18 hr. After washing the bacterial cells in plant culture medium, we inoculated plant cells with induced Agrobacterium (~20 bacterial cells per plant cell, except where noted otherwise) and cocultivated the cells at 25°C with shaking at 140 rpm for various periods of time. We washed off most of the bacteria by centrifugation of the cocultivation mixture at 300 rpm (model GLC-2 clinical centrifuge; Beckman Sorvall, Newtown, CT) for 2 min. The plant cell pellet was suspended and washed once more in plant culture medium and then resuspended in culture containing either 100 μg/mL timentin or 200 μg/mL cefotaxime. To collect plant cells for isolation of RNA, we washed the cells three times, as described above, in plant culture medium. We extracted RNA from these cells either directly after harvesting (either of the two methods listed below) or after freezing in liquid nitrogen and storage at −70°C (TRIzol reagent [Gibco BRL] extraction method).

To determine the percentage of cells expressing GUS activity, the cells were incubated in GUS histochemical staining solution (50 mM NaH_2PO_4, 10 mM Na_2·EDTA, 0.3 M mannitol, 20% methanol, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid [X-gluc]) overnight at 37°C (Kosuge et al., 1990).

Isolation of Plant RNA and Reverse Transcriptase–Polymerase Chain Reaction

After sonication of the cells, we isolated RNA by using the GlassMAX system (Gibco BRL), according to the instructions of the supplier. Alternatively, after freezing the cells in liquid nitrogen, we isolated RNA by using the TRIzol reagent system according to the instructions of the supplier, with modifications (as described in the Bethesda Research Laboratory corporate publication Focus **17,** 20–21). The TRIzol reagent method yielded considerably more RNA than the alternate method. We treated the nucleic acids with RNase-free DNase I (1 unit per μg; Gibco BRL) and then heat-inactivated DNase I and subjected the resulting RNA (1 μg) to reverse transcription using a reverse transcription system (Promega) according to the instructions of the supplier. We used oligo (dT) to prime cDNA synthesis.

We amplified *gusA* sequences in a 50- or 100-μL reaction volume by using Taq DNA polymerase and the primers 5′-ACGATCAGTTCGCCGATGG-3′ (forward primer) and 5′-TCCCGCTAGTGCCTTGTCC-3′ (reverse primer). The amplification was conducted for 40 cycles with the following program: 94°C for 2 min (one cycle); 92°C for 40 sec,

53°C for 1 min, and 72°C for 2 min (40 cycles); and 72°C for 5 min (one cycle). We analyzed 10- to 15-μL samples by electrophoresis through a 1.5% agarose gel. DNA gel blot hybridization, using a *gusA* gene probe, verified that the amplified bands represented *gusA* sequences (data not shown).

Quantitation of *gusA* mRNA

We quantitated *gusA* mRNA by adding a known quantity of in vitro–transcribed *gusA*-intron RNA to the reverse transcriptase–polymerase chain reaction (RT-PCR) containing plant RNA. To generate the *gusA*-intron template for in vitro transcription, we first cloned an EcoRI (made blunt by using the Klenow fragment)–SalI fragment (containing the *gusA*-intron gene) into the SmaI and SalI sites of pSP64(polyA) (Promega) to generate pE1287. We next cloned a SalI-EcoRI fragment from pE1287 into pBluescript SK+ to generate pE1288. To generate in vitro *gusA*-intron transcripts from pE1288, we used T7 RNA polymerase and an in vitro transcription kit (Stratagene) according to the specifications of the supplier. We mixed various amounts of in vitro–synthesized *gusA*-intron RNA with 1 μg of plant RNA and subjected the mixture to RT-PCR as described above, including the use of oligo(dT) to prime cDNA synthesis. After electrophoresis of the amplified products, we compared the signal intensity derived from the *gusA*-intron RNA (732 bp) with that derived from the processed *gusA* mRNA (543 bp). We quantitated the amount of processed *gusA* mRNA in the plant samples by comparison with the amount of *gusA*-intron RNA that yielded a signal of the same intensity.

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