MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 5, pp. 500-506, 1991

Efficient Splicing of the *Tetrahymena* Group I Intron in Transformed Tobacco Plants: Further Evidence for DNA to DNA Information Flow in Transformation by *Agrobacterium tumefaciens*

Gail Dinter-Gottlieb, Tzion Fahima, and Nadine Pobjecky

Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104 U.S.A.
Received 29 November 1990. Revised 10 April 1991. Accepted 30 April 1991.

We have used the self-splicing group I intron from *Tetrahymena thermophila* rRNA under control of the cauliflower mosaic virus (CaMV) 35S promoter to follow transformation of tobacco plants by the T-DNA of *Agrobacterium tumefaciens*. The presence of a potentially self-splicing RNA intermediate in the transformation process would allow splicing of the intron to occur, and the intron sequence would be absent from the plasmid integrated in the plant DNA. Assay of the integrated DNA demonstrated that in all cases, regardless of intron orientation, the intron cDNA was present in the plant. In constructions in which the intron precursor RNA under control of the CaMV promoter was in the correct orientation, efficient splicing of the intron from the precursor RNA occurred in the plant. This supports the view that T-DNA transformation involves a DNA to DNA transfer, without an RNA intermediate. It also demonstrates that efficient splicing of a nuclear ribosomal RNA intron from a protozoan can occur in a plant milieu.

Additional keywords: Agrobacterium transformation.

Transformation of plant cells by *Agrobacterium tumefaciens* (Smith and Townsend) Conn Ti plasmid involves the transfer of a copy of a portion of the plasmid DNA, termed the T-DNA (see Koukolikova-Nicola *et al.* 1987; Nester *et al.* 1984; Stachel and Zambryski 1986, for review). T-DNA transfer initiates with induction of the plasmid *vir* gene locus by plant signal molecules (Stachel and Zambryski 1986), resulting in production of a single-stranded DNA molecule (T-strand), homologous to the bottom strand of the T-DNA (Stachel *et al.* 1986; Stachel *et al.* 1987). This T-strand DNA, which has been shown to be associated with single-stranded DNA-binding proteins (Citovsky *et al.* 1988; Geital *et al.* 1987), is presumably the intermediate for information transfer between the *Agrobacterium* and the plant. The details of subsequent transfer and integration into the plant DNA have not been defined. Further, although double-stranded linear and circular T-DNA molecules have been detected (Jayaswal *et al.* 1987; Koukolikova-Nicola *et al.* 1985), a rigorous search for a possible RNA intermediate has not been reported.

Intron excision detected an unsuspected RNA intermediate in yeast transposition by a Ty element (Boeke *et al.* 1985) and was used to define an RNA to DNA step in replication of cauliflower mosaic virus replication (Hohn *et al.* 1986). In these studies, a messenger RNA intron was used, and its splicing depended on the localization of the intermediate RNA at some point in the nucleus of the plant or yeast cell in order for the intron to be accessible to the normal splicing components. Recently Vancanneyt *et al.* (1990) inserted a plant intron into the β-glucuronidase (GUS) gene and assayed β-glucuronidase expression in *Agrobacterium* and in the plant. The presence of the intron prevented GUS expression in the bacteria, because no prokaryotic mechanism exists for intron excision, but the intron was absent from the plant RNA. They did not, however, assay the plant DNA to determine if the intron was present in the plant after transformation.

We have used the 23S ribosomal RNA intron (IVS) from *Tetrahymena thermophila*, under control of a cauliflower mosaic virus (CaMV) 35S promoter, inserted upstream of the gene for β-glucuronidase expression, as a marker for passage through an RNA intermediate. This intron has the ability to self-splice *in vitro* (Kruger *et al.* 1982) and was correctly excised from RNA products of plasmid or phage in *E. coli* (Waring *et al.* 1985; Price and Cech 1985). Thus, if at any point in the transfer and integration process, the T-DNA passed through an RNA intermediate, regardless of whether the RNA was in the nucleus, intron splicing could conceivably occur. The integrated DNA would then be lacking the intron sequence. Introducing the intron in both orientations would allow transcription of an intron of self-splicing polarity no matter which RNA strand might be the intermediate.

The data show that, in all cases, the intron sequence was present in the integrated DNA of the plant. The resultant GUS mRNA expressed in the plant was precisely and efficiently spliced when the intron RNA was in positive orientation. The transfer of information from *Agrobacterium* to plant apparently involves a DNA to DNA information flow, with no RNA intermediate. Furthermore, accurate and efficient splicing of the *Tetrahymena* group I intron occurred in the plant cell.

MATERIALS AND METHODS

Construction of pCIG4 and pCIG12. DNA manipulations were performed essentially as described in Maniatis *et al.* (1982). The *Tetrahymena* IVS from pBMG (Welch 1989) was excised as an *Eco*RI/*Hin*dIII fragment, containing 467 bp. The 413-bp IVS was flanked by a 29-bp 5′ exon and a 25-bp 3′ exon. The fragment was subcloned into a plant expression vector, pBI121 obtained from R. Jefferson (1987), between the 35S CaMV promoter and the GUS gene, in both orientations. The final constructions of the plasmids are shown in Figure 1. Plasmid pBI121 containing only the CaMV promoter and GUS gene, was used as a control. The orientations were confirmed by sequencing (data not shown).

***Agrobacterium*-mediated transformation of tobacco leaf disks.** The plasmids, pCIG4, pCIG12, and pBI121, in *E. coli* DH5α, were mobilized into the *Agrobacterium* strain LBA4404 (Ooms *et al.* 1981) by the triparental mating procedure with *E. coli* HB101 carrying pRK2013 (Bevan 1984). Transconjugants were checked by drug resistance and used to transform tobacco cells (*Nicotiana tabacum* 'Samsun') by the leaf-disk cocultivation method (Horsch *et al.* 1985). Clones of transgenic plants were obtained by regeneration of shoots from leaf disks under selective pressure (100 μg/ml kanamycin). Rooted transformants were transferred to soil and grown to maturity under standard greenhouse conditions. A second set of controls, in addition to the plants transformed with pBI121, consisted of tobacco leaf disks that were not transformed. These showed no regeneration on plates containing kanamycin.

Segregation analysis. Transformed plants were self-pollinated and the resulting seeds were plated on MS medium (Murashige and Skoog 1962) containing 100 μg/ml kanamycin. Seedlings were scored after 2–3 wk as either sensitive or resistant.

Assay for β-glucuronidase activity in the transformed plants. The expression of the gene for β-glucuronidase (GUS) was assayed by the method of Jefferson *et al.* (1987). Leaf tissue from transformed plants was homogenized with lysis buffer, and the fluorometric reaction was carried out in 1 mM 4-methylumbelliferyl glucuronide in lysis buffer. The fluorescence emitted was then measured at 455 nm in a spectrofluorometer.

Polymerase chain reaction (PCR) analysis of genomic DNA. Total plant DNA was isolated from all Km^r transformants by the method of Junghans and Metzlaff (1990). PCR reactions were carried out using plant DNA prepared as described below as template DNA. Genomic DNA (0.2–0.5 μg) was mixed with 25–50 pmol of each of a pair of specific primers (see Fig. 2A) and amplified with Taq (*T. aquaticus*) polymerase (Saiki 1985) using the Gene-Amp DNA amplification kit (Cetus, Norwalk, CT) in a DNA thermal cycler (Coy Laboratory Products Inc., Ann Arbor, MI). Amplification conditions consisted of an initial incubation at 94° C for 2 min, followed by 35 cycles of denaturation (1 min at 94° C), annealing (2 min at 50° C), and polymerization for 2 min at 72° C. A 10-μl sample of the reaction was then removed and analyzed on a composite agarose gel (2% Nusieve, 0.5 Seakem ME; FMC Corporation, Rockland, ME). The bands were visualized by ultraviolet illumination.

Probing of amplified DNA. The separated bands of DNA were denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized with 1.5 M NaCl, 0.5 M Tris, pH 7.0, and blotted onto nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Richmond, CA). The membranes were then hybridized with a primer 5′ end-labeled with [γ-^{32}P]ATP, using polynucleotide kinase. The 17-nt primer hybridized at position 274–290 on the intron sequence (Welch 1989). After hybridization for 18 h at 42° C in 50% formamide, the membrane was washed for times in SSC buffer (5×, 2×, 1×, and

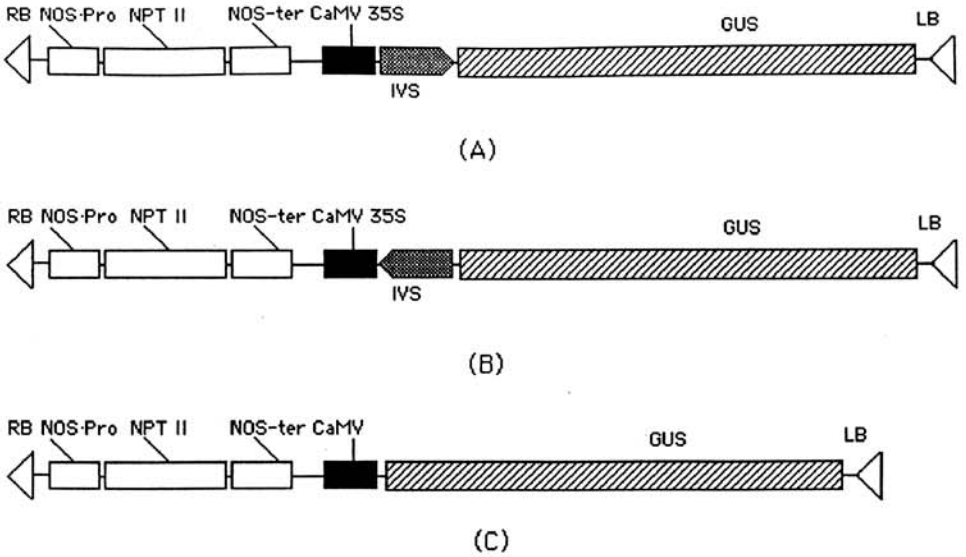

Fig. 1. Construction of the plasmids used in tobacco leaf disk transformation. **A,** pCIG4, in which the *Tetrahymena* intron (IVS) is in positive orientation with the cauliflower mosaic virus (CaMV) promoter. **B,** pCIG12 carries the IVS insert in reverse orientation, so that transcription from the CaMV promoter will produce an "anti-intron." On the other hand, in an RNA intermediate of this polarity, the intron could splice in transit. **C,** pBI121, a control plasmid not carrying the intron cDNA.

and 0.5×; 1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate), and 0.1% sodium dodecyl sulfate, at room temperature for 20 min, and exposed for autoradiography (Selden 1989).

RNA isolation. Total RNA was isolated from leaf material by the method of Thompson *et al.* (1983).

Reverse transcription of plant RNA. Total leaf RNA (5 μg) was primed using a synthetic oligonucleotide of 20 nts (Fig. 3A, primer 2), and cDNA was synthesized using AMV reverse transcriptase according to Frohman *et al.* (1988). A 5-μl sample of the reaction mixture was then removed, diluted to 250 μl with water and stored at −20° C.

PCR amplification of the cDNA. Amplification of the cDNA derived from total plant RNA was carried out as described above for the genomic DNA. A 20-μl sample of the diluted cDNA mixture was taken (see above), and 50 pmol of both primers were added (Fig. 3A, primers 2 and 3). Samples of 10 μl of amplified products were analyzed in an 8% polyacrylamide gel, stained with ethidium bromide and photographed (Fig. 2B).

Sequencing of the PCR products. Amplified DNA products were extracted with phenol and chloroform and were

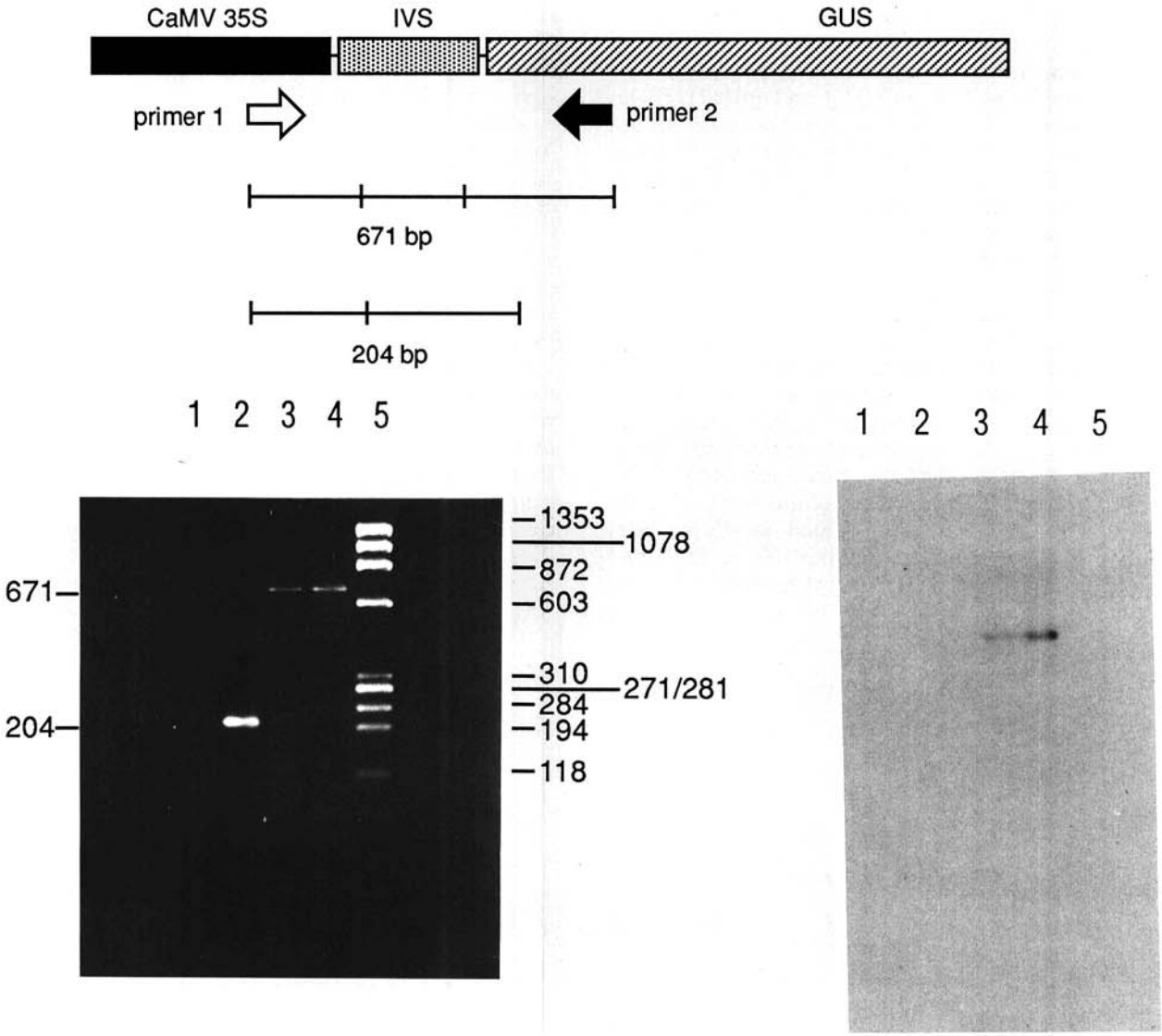

Fig. 2. Strategy for assaying the plant DNA for the presence of the IVS sequences. Top, the assay depends on the presence of the cauliflower mosaic virus (CaMV) promoter and the GUS gene, because the primer DNA oligonucleotides are complementary to those regions of the DNA. Primer 1 had the sequence 5′-TCACGGGTTGGGGTTTCTAC and was complementary to the CaMV 35S promoter, whereas primer 2, complementary to the GUS gene, was 5′-ACGTTCCAACCACGTGTTCAAAGCAAGTGG. The size of the product defines the presence of the IVS in the DNA integrated into the plant. Bottom left, an agarose gel analysis of the products obtained from the plant DNA amplification. Lane 1, DNA from an untransformed plant (160); lane 2, amplified DNA from a plant transformed with pBI121 (106). The band is 204 bp in length, since no intron or flanking exons sequences are present. Lane 3, DNA from (72), transformed with pCIG4. The 671-bp band indicates the IVS remained in the DNA. Lane 4, pCIG12 transformed plant DNA from (156) shows the presence of the IVS and exons. Lane 5, *Hae*III cut φ174 marker DNA. Bottom right, confirmation of the identity of the fragments by Southern blotting and hybridization with IVS-specific labeled DNA probes. The gel shown in Figure 3B was blotted and probed with a 17-nt oligonucleotide complementary to the *Tetrahymena* intron (Welch 1989). Lanes 3 and 4 show the presence of the IVS in both orientations.

electrophoresed on an 8% polyacrylamide gel. The product bands were excised, and the DNA was eluted and recovered by ethanol precipitation. The sequencing primer was 5′ end-labeled with [γ-^{32}P]ATP using polynucleotide kinase (Promega Corporation, Madison, WI). Amplified DNA (0.5–1.0 pmol) and primer (1.0 pmol) were annealed as described by Kusukawa *et al.* (1990), and sequenced with the Taq-track sequencing system (Promega).

RESULTS

Construction of intron-containing plasmids. The *T. thermophila* intron, with flanking exons, was cloned into a unique *Sma*I site in plasmid pBI121 between the 35S CaMV promoter and the gene for β-glucuronidase (Fig. 1). The intron was cloned into both orientations: pCIG4 would produce a GUS mRNA transcript with the intron upstream in positive orientation, capable of self-splicing, while pCIG12 contained the intron in reverse orientation, so that the GUS mRNA product would contain an "anti-intron," incapable of self-splicing. Although the T-strand DNA has been shown to be homologous to the bottom strand of the T-region, that is, the 5′ and 3′ ends mapped to the right and left T-DNA borders (Stachel and Zambryski 1986), an RNA intermediate could conceivably be of either polarity, depending on when it arose in the cycle. Therefore the intron RNA could be transcribed in positive orientation regardless of which strand served as an intermediate. On the other hand, in terms of splicing of the integrated GUS gene mRNA product, only the pCIG4 RNA would be expected to splice. The orientation of the intron sequences was confirmed by DNA sequencing (data not shown).

Tobacco leaf disk transformation and assay for GUS. Transformation of tobacco leaf disks was carried out as described (see Methods). The transformants were selected on medium containing 100 μg/ml kanamycin, and assayed for GUS gene expression, using the fluorimetric assay (Jefferson *et al.* 1987) described in Methods. As seen in Table 1, not all the transformed plants selected for kanamycin resistance were positive for GUS expression. Although expression of the GUS gene served as an indicator of transformation, GUS gene expression did not depend on intron excision, since the initiation codon for translation was downstream of the inserted intron. Lack of GUS gene expression might result from alteration of the promoter or other signals during the transformation or integration process or, since the GUS gene was distal to the Km gene with respect to the right border, it is possible that only a portion of the GUS gene was integrated. The regenerated plants resistant to kanamycin were assayed for presence of the intron, regardless of whether GUS expression was detected. Kanamycin-resistant plants showed a 3:1 segregation ratio of Km^r to Km^s in the progeny of self-pollinated plants, indicating that integration of the construct into the plant DNA had occurred.

Assay of the transformed plant DNA using the polymerase chain reaction (PCR). To detect the presence of the intron in the DNA of transformed plants, a strategy using the polymerase chain reaction was employed. Two DNA oligonucleotide primers were designed as probes for the inserted DNA, flanking the site of insertion of the intron (Fig. 2A). If the intron, in either orientation, were present in the integrated DNA, the resulting fragment would be 671 bp in length. Intron excision during passage through an RNA intermediate would result in an amplified fragment of 237 bp in length, since the flanking exon sequences would remain behind and be integrated into the DNA. The pBI121 control would produce a 183-bp fragment. As seen in Table 1, the DNA from all the plants transformed with pCIG4 or pCIG12 contained a fragment of 671 bp (Fig. 2B), indicating that the intron was present in the DNA. The control plants transformed with pBI121, which contained only the GUS gene showed a 183-bp fragment, as expected. The controls that were not transformed showed no amplified DNA product (Table 1). Southern blotting of the DNA fragments with an oligonucleotide complementary to the

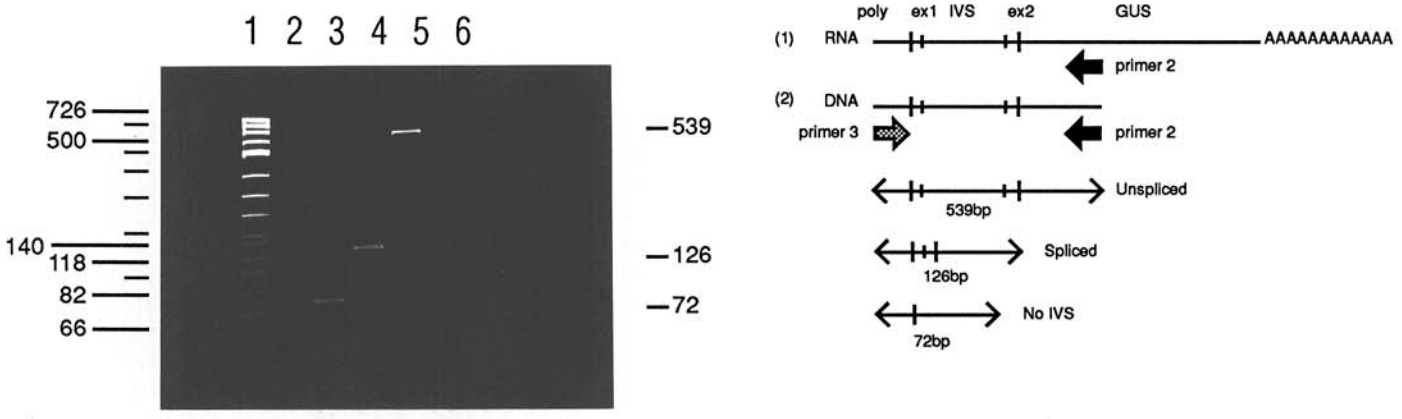

Fig. 3. Analysis of the plant RNA for intron splicing, using reverse transcription followed by polymerase chain reaction amplification. Right, primer 2 was the same as utilized for the DNA, since it was complementary to the GUS gene mRNA. Primer 3 was complementary to the polylinker region, and read 5′-CACGGGGGACTCTAGAGGATCCCC. The three possible DNA products resulting from PCR amplification are diagrammed. Splicing of the IVS would still leave the ligated exon nucleotides behind. Left, the PCR products on an agarose sizing gel. The size of the DNA fragments indicated that complete splicing of the RNA had occurred if the intron was in positive orientation with the promoter, transformed with pCIG4 (Lane 4, plant 125). Lane 1 shows *Hin*fI cut ϕX 174 marker DNA, lane 2 the result of amplication of RNA from an untransformed plant (160), lane 3 the RNA from a plant transformed with an intron-less construct, pBI121 (103), and Lane 5 the pCIG12 RNA containing the intron in reverse orientation, unspliced (156). Lane 6 is a "false primed" control, in which primers 1 and 2 were used to detect contaminating genomic DNA (156). None was present, as shown by absence of any band.

intron confirmed the presence of the intron in all pCIG4 and pCIG12 plants (Fig. 2C). The data implied that no RNA intermediate was involved in the transformation. The possibility remained, however, that the intron could not be spliced from an RNA intermediate, either because the RNA was deficient in self-splicing activity, or because some plant product inhibited the splicing reaction. We thus assayed the GUS mRNA products for the presence of the intron.

PCR assay for RNA splicing of the GUS mRNA. Total plant RNA was isolated and copied into cDNA by use of reverse transcriptase (see Methods), and the DNA products were then amplified by the PCR method using specific sets of DNA oligonucleotide primers (Fig. 3A). The first primer (primer 2) was designed to initiate copying of the single-stranded cDNA transcribed from the RNA, and to hybridize within the GUS gene. The second primer (primer 3) would hybridize within the polylinker region derived from the 5′ terminus of the transcribed RNA. If the RNA was unspliced, a PCR product of 539 bp would result. If the RNA was spliced and the IVS was properly excised, a 126-bp product would be detected. Partial splicing would result in products of both sizes. The control plants, transformed with pBI121, without the IVS insertion, would yield a 72-bp product.

In those plants transformed with pCIG4, in which the intron was in positive orientation in reference to the CaMV promoter, the only DNA product detected was 126-bp in length, indicating that splicing of the intron from the precursor RNA had occurred (Fig. 3B). The residual 54 nts of exon RNA sequences remained after splicing. In the plants transformed with pCIG12, the resulting fragment was 539 bp in length, since the intron in negative orientation and its flanking exons were still present in the RNA product (Fig. 3B and Table 1). The control pBI121-transformed plants showed a 72-bp fragment.

To confirm the precision of the splicing event, the DNA generated from the pCIG4 and pCIG12 plants was sequenced using primer extension (see Methods). The sequence of the spliced product, CUCUCU|UAAG, with the splice junction at the slash, revealed that splicing was precisely as expected from a self-splicing reaction, between a U|A junction at the 5′ end and a G|U at the 3′ terminus (data not shown). This indicated that the intron was properly excised and the exons ligated during the splicing reaction. Although an *in vivo* splicing reaction cannot be strictly termed "self-splicing," it is clear that a splicing reaction identical to the *in vitro* self-splicing occurred in the plant. Further, the splicing reaction was rapid and efficient, because no precursor RNA was detected in the pCIG4 plants, even after PCR amplification. Thus, all the pCIG4 GUS mRNA was rapidly and accurately spliced. On the other hand, the pCIG12 "anti-intron" RNA remained within the precursor, neither capable of self-splicing nor of being spliced by external factors.

Table 1. Transformation of tobacco leaf disks with *Agrobacterium tumefaciens* T-DNA and analysis of plant DNA and mRNA for presence of the *Tetrahymena* intron

	Plasmid	Plant no.	GUS activity	PCR DNA size	PCR RNA size
GUS gene only	pBI121	101	+	204	72
		102	−	204	ND
		103	+	204	72
		104	+	204	ND
		106	+	204	ND
Intron (+)	pCIG4	120	+	671	126
		121	+	671	126
		122	+	671	126
		123	−	671	126
		125	+	671	126
		127	+	671	126
		128	+	671	126
		130	−	671	126
		132	+	671	126
Intron (−)	pCIG12	140	+	671	539
		142	+	671	539
		144	+	671	539
		145	−	671	539
		146	+	671	539
		152	−	671	539
		153	+	671	539
		154	−	671	539
		156	+	671	539
		158	−	671	539
Control	None	160	−	−	+
		162	−	−	+
		163	−	−	+
		164	−	−	+

[a] Not determined.

DISCUSSION

The intron cDNA is integrated in every transformed plant DNA. Regardless of initial orientation, the intron cDNA was detected by PCR amplification in all the plants transformed with either pCIG4 or pCIG12. These plants were also capable of GUS gene expression, as indicated by fluorometric assay for GUS gene product. The presence of the intron indicates that either the transformation into the plant involved only DNA to DNA transfer, or, if an RNA intermediate was involved, the intron could not be spliced from the RNA. This might occur if the intron was defective, if self-splicing cannot occur in the plant milieu, or if the splicing was blocked by other factors. None of the above appear to be true, however, because the RNA products from the integrated pCIG4 plasmid were completely and accurately spliced. No unspliced pCIG4 RNA product could be detected, even through PCR amplification. This indicated that when the intron precursor RNA was present, highly efficient splicing could occur. Only if the RNA intermediate consisted solely of the pCIG12 "anti-intron" RNA would splicing be unable to occur. This possibility was controlled for by using both orientations of intron, so that an intron of positive orientation would be produced no matter which DNA strand served as a template.

The advantage of using the self-splicing intron as a marker lies in its ability to splice outside the nucleus and its independence from any nuclear splicing mechanism. Thus, even if an RNA intermediate was formed in the *A. tumefaciens* before the plant transfer, the splicing reaction

could occur. This has been shown using the same intron in *E. coli* (Waring *et al.* 1985; Price and Cech 1985). It is impossible to rule out the possibility that an RNA intermediate was too short-lived to splice, or was prevented from splicing by its location or by suppressor molecules in the plant, but the efficient splicing of the intron from the GUS mRNA makes these hypotheses unlikely.

Accurate splicing of a group I intron contained in a mRNA occurs in tobacco plants. A group I intron, highly homologous in sequence to the *T. thermophila* intron, has been identified in the chloroplast $tRNA^{leu}$ genes for maize, broad bean, and tobacco (Bonnard *et al.* 1984; Steinmetz *et al.* 1982; Yamada *et al.* 1986). It is interesting that the precursor RNAs transcribed from the cloned tRNA genes appear to be incapable of self-splicing *in vitro* (P. Gegenheimer, personal communication). It may be that these chloroplast introns, similar to the *Neurospora crassa* rRNA group I intron (Akins and Lambowitz 1987), require protein factors found in the chloroplast for their splicing. In the experiments described here, the *Tetrahymena* intron was spliced correctly in the plant, although the location of the splicing event has not been identified. It remains to be determined whether a true self-splicing occurred in the plant cell, or whether plant proteins were involved in the splicing.

These data support the view that a DNA to DNA transfer, possibly analogous to bacterial conjugation (Stachel and Zambryski 1986) or transposition, is operant in *A. tumefaciens* transformation. A DNA-RNA-DNA transfer, as seen with the yeast Ty transposon, retroviruses, Hepatitis B, and CaMV (Baltimore 1970; Pfeiffer and Hohn 1983; Summers and Mason 1982; Temin 1970) should have resulted in loss of the intron from at least some of the transformed DNA. This was not detected. The data further indicate that efficient and accurate splicing of the nuclear rRNA intron from *T. thermophila* can occur in a tobacco plant cell.

ACKNOWLEDGMENTS

We thank Andy Binns for technical help and encouragement, and Tony Volker, Richard Jefferson, and Michael Dobres for technical information and helpful discussions. The efforts of Jan Sharp in initiating the project and the encouragement of T. R. Cech are acknowledged. This research was supported by grant MV-421 from the American Cancer Society. T. F. was the recipient of a Lady Davis Fellowship, Jerusalem, Israel 91904.

LITERATURE CITED

Akins, R. A., and Lambowitz, A. M. 1987. A protein required for splicing group I introns in *Neurospora* mitochondria is mitochondrial tyrosyl-tRNA synthetase or a derivative thereof. Cell 50:331-345.

Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature (London) 226:1209-1211.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711-8721.

Boeke, J. D., Garfinkel, D. J., Styles C. A., and Fink, G. R. 1985. Ty elements transpose through an RNA intermediate. Cell 40:491-500.

Bonnard, G., Michel, F., Weil, J. H., and Steinmetz, A. 1984. Nucleotide sequence of the split $tRNA^{leu}$ UAA gene from *Vicia faba* chloroplasts: Evidence for structural homologies of the chloroplast $tRNA^{leu}$ intron with the intron from the autosplicable *Tetrahymena* ribosomal RNA precursor. Mol. Gen. Genet. 194:330-336.

Citovsky, V., DeVos, G., and Zambryski, P. C. 1988. Single-stranded DNA binding protein encoded by the vir E locus of *Agrobacterium tumefaciens*. Science 240:501-504.

Frohman, M. A., Dush, M. K., and Martin, G. R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85:8998-9002.

Geitl, C., Koukolikova-Nicola, Z., and Hohn, B. 1987. Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA. Proc. Natl. Acad. Sci. USA 84:9006-9010.

Hohn, B., Balazs, E., Ruegg, D., and Hohn, T. 1986. Splicing of an intervening sequence from hybrid cauliflower mosaic viral RNA. EMBO J. 5:2759-2762.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. D., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231.

Jayaswal, R. K., Veluthambi, K., Gelvin, S., and Slightom, J. L. 1987. Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in *Escherichia coli* by a vir D-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. J. Bacteriol. 169:5035-5045.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS-fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Plant Mol. Biol. Rep. 5:387-405.

Junghans, H., and Metzlaff, M. 1990. A simple and rapid method for the preparation of total plant DNA. Biotechniques 8:176.

Koukolikova-Nicola, Z., Albright, L., and Hohn, B. 1987. Plant DNA infectious agents. Pages 110-148 in: Plant Gene Research. Springer-Verlag, Vienna.

Koukolikova-Nicola, Z., Shillito, R. D., Hohn, B., Wang, K., Van Montagu, M., and Zambryski, P. C. 1985. Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells. Nature (London) 313:191-196.

Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E., and Cech, T. R. 1982. Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. Cell 31:147-157.

Kusukawa, N., Uemori, T., Asada, K., and Kato, I. 1990. Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. Biotechnology 9:66-72.

Maniatis, T. A., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:4467-4497.

Nester, E. W., Gordon, M. P., Amasino, R. M., and Yanofsky, M. F. 1984. Crown gall: A molecular and physiological analysis. Annu. Rev. Plant Physiol. 35:387-413.

Ooms, G., Hooykas, P. J. J., Moolenaar, M., and Schilpoort, A. 1981. Crown gall plant tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying octopine Ti plasmids: Analysis of T-DNA function. Gene 14:33-50.

Pfeiffer, P., and Hohn, T. 1983. Involvement of reverse transcription in the replication of cauliflower mosaic virus: A detailed model and test of some aspects. Cell 33:781-789.

Price, J. V., and Cech, T. R. 1985. Coupling of *Tetrahymena* ribosomal RNA splicing to β-galactosidase expression in *Escherichia coli*. Science 228:719-722.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Schart, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Ehrlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 240:487-491.

Selden, R. F. 1989. Analysis of DNA sequences by blotting and hybridization. Pages 291-299 In: Current Protocals in Molecular Biology. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. John Wiley & Sons, New York.

Stachel, S. E., Timmerman, B., and Zambryski, P. C. 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature (London) 322:706-721.

Stachel, S. E., Timmerman, B., and Zambryski, P. C. 1987. Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: Requirement for 5′ virD gene products. EMBO J. 6:857-863.

Stachel, S. E., and Zambryski, P. C. 1986. *Agrobacterium tumefaciens* and the susceptible plant cell: A novel adaptation of extracellular

recognition and DNA conjugation. Cell 47:155-157.
Steinmetz, A., Gubbins, E. J., and Bogorad, L. 1982. The anticodon of the maize chloroplast gene for $tRNA^{leu}$ UAA is split by a large intron. Nucleic Acids Res. 10:3027-3037.
Summers, J., and Mason, W. S. 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29:403-415.
Temin, H. M. 1970. RNA-directed DNA polymerase in virions from *Rous sarcoma* virus. Nature (London) 226:1211-1213.
Thompson, W. F., Everett, E., Polans, N. O., Jorgenson, R. A., and Palmer, J. O. 1983. Phytochrome control of RNA levels in developing pea and mung bean leaves. Planta 158:487-500.
Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L., and Rocha-Sosa, M. 1990. Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol. Gen. Genet. 220:245-250.
Waring, R. B., Ray, J. A., Edwards, S. W., Scazzocchio, C., and Davies, R. W. 1985. The *Tetrahymena* rRNA intron self-splices in *E. coli*: *In vivo* evidence for the importance of key base-paired regions of RNA for RNA enzyme function. Cell 40:371-380.
Welch, A. 1989. Site-directed mutagenesis of the *Tetrahymena thermophila* rRNA "self-splicing" intron. M. S. thesis. Drexel University, Philadelphia, PA.
Yamada, K., Shinozaki, K., and Suguira, M. 1986. DNA sequences of tobacco chloroplast genes for $tRNA^{Thr}$(UGU), $tRNA^{Leu}$(UAA), $tRNA^{Leu}$(GAA): The $tRNA^{Leu}$ gene contains a 503-bp intron. Plant Mol. Biol. 6:193-199.