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The expression of a chimeric soybean beta-tubulin gene in tobacco

Mark J. Guiltinan[1] **, Jeff Velten[2] *, Mauricio M. Bustos[1] **, Richard J. Cyr[1] *, Jeff Schell[2], and Donald E. Fosket[1]**

[1] University of California, Irvine, CA 92717, USA

[2] Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, Federal Republic of Germany

Summary. A chimeric tubulin gene has been constructed by the fusion of a genomic sequence containing a truncated soybean beta-tubulin gene with 2 kb of upstream DNA to the 3′ untranslated region containing the polyadenylation signal from transcription unit 7 of the octopine Ti plasmid pGV117. The chimeric gene has been incorporated into the Ti plasmid transformation vector pGV3850::pAP2034, along with a selectable marker gene active in plants (the neomycin phosphotransferase II gene, conferring kanamycin resistance). Strains of *Agrobacterium tumefaciens* harboring the plasmids were used to transform *Nicotiana tabacum* by the leaf disk method and plants were regenerated from transformed cells. Transgenic plants were self fertilized and the segregation of kanamycin resistance was assayed. DNA and RNA were extracted from transgenic plants, fractionated by agarose gel electrophoresis, blotted to nitrocellulose and hybridized to a probe specific for the chimeric gene to assess its structure and expression in transgenic plants. The chimeric gene was stably integrated into the tobacco genome without rearrangements and it was expressed as a polyadenylated RNA of 1.7 kb in the transformants. Genetic analysis revealed that the kanamycin-resistant phenotype was inherited in a Mendelian fashion over two generations.

Key words: Tubulin – Cell transformation – Gene expression – Ti plasmid – *Agrobacterium tumefaciens*

Introduction

Microtubules are composed principally of the dimeric protein tubulin whose subunit polypeptides, designated alpha- and beta-tubulin, have molecular weights of about 50 kilodaltons (kDa) (Ponstingle et al. 1981; Krauhs et al. 1981). Tubulin is found in the cell as a soluble dimer or polymerized to form microtubules. Frequently these two states are in a dynamic equilibrium which can be altered by a number of factors, including Ca^{2+}, temperature, [GTP], microtubule associated proteins (MAPS) and several tubulin-binding ligands such as colchicine and taxol (Kirschner 1978). Multiple isoforms of alpha- and beta-tubulin have been observed in most species examined. In some cases, these isoforms arise by post-translational modifications while in other cases, specific tubulin isoforms are the products of different tubulin genes (see review, Cleveland and Sullivan 1985).

The tubulins are encoded by multigene families in all eukaryotes examined thus far, except *Saccharomyces cerevisiae* and *Tetrahymena* which each have only one beta-tubulin gene (Cleveland and Sullivan 1985). Comparison of sequence data from 15 different beta-tubulin genes from a wide variety of organisms indicates that their amino acid sequences are highly conserved, and that the divergence between isotypes is clustered primarily in one highly variable domain (amino acid positions 430 to the C-terminus (Cleveland and Sullivan 1985). A highly conserved region also can be identified (amino acid positions 401–425) in which there is nearly 100% sequence conservation between the divergent species. Interspecific conservation of the variable domains and 3′ untranslated sequences of several vertebrate tubulin genes indicates strong evolutionary pressure to maintain specific isotypes and untranslated sequences within a species (Cowan et al. 1983). Since microtubules have mutiple functions in vivo, the individual tubulin isotypes may have some degree of functional specificity. Although in a few cases there is evidence that individual tubulin isotypes may have a specific function (Weatherbee et al. 1985; Burland et al. 1983), the significance of the multiplicity of tubulin genes in most species is not known. A complete understanding of the functional relationships between the different members of the tubulin gene families is essential to our knowledge of the fundamental mechanisms of cellular growth and development of all eukaryotic organisms.

To begin a study of the organization and expression of tubulin genes in higher plants, we have isolated several genomic clones containing tubulin sequences from a soybean library (kindly provided by Dr. Robert Goldberg, UCLA). These were characterized by restriction mapping, hybridization analysis and DNA sequencing and they have been used as probes for the analysis of tubulin mRNA levels in various plant tissues (Guiltinan et al. 1986; Cyr et al. 1986). The soybean haploid genome contains two beta-tubulin genes (designated SB-1 and SB-2). Partial sequence data from these clones shows that, although the

* *Present address:* New Mexico State University, Las Cruces, NM 88003, USA

** *Present address:* Texas A&M University, College Station, TX 77843-3258, USA

*** *Present address:* University of Georgia, Athens, GA 30602, USA

Offprint requests to: D.E. Fosket

predicted amino acid sequences of the carboxy termini from the soybean tubulin genes are homologous to beta-tubulins from other species (62%–80% homology), these sequences are significantly different from each other and are the second (after yeast) most highly diverged beta-tubulin genes thus far examined. Several size classes of beta-tubulin poly(A)$^+$ RNA are present in soybean seedlings and the cellular levels of these transcripts are modulated both quantitatively and qualitatively during development (Bustos et al. 1986).

As an initial approach towards determining the factors responsible for the developmental regulation of tubulin genes, we have cloned a chimeric beta-tubulin gene into the T-DNA of the Ti plasmid vector pGV3850::pAP2034 (Zambryski et al. 1983; Velten and Schell 1985). This construct was used to transform tobacco cells which were then regenerated into fertile plants. We report here that the soybean beta-1-tubulin gene was stably integrated into the tobacco genome and inherited in a Mendelian fashion through several generations. The foreign gene is expressed as poly(A)$^+$ RNA in the transgenic plants.

Materials and methods

Bacterial strains, plasmids and plant lines. The strains of *Escherichia coli* used for plasmid cloning were JM103 for M13 plasmids (Yanisch-Perron et al. 1985) and TB1 (kindly provided by T. Baldwin, Texas A&M University) for pBR322 derivatives pUC13 and SP6. Conjugation between *E. coli* TB1 harboring derivatives of the plasmid pAP2034 (Velten and Schell 1985), and *E. coli* strain GJ23 containing the plasmids R64drd11 and pGJ28 was performed according to Van Haute et al. (1983). The resulting strains were conjugated to *Agrobacterium tumefaciens* strain C58C1 (rif) (Zambryski et al. 1983) containing the disarmed Ti plasmid pGV3850. Transconjugants were used to transform leaf disks of *N. tabacum* Petit Havana cv. SR1 (Horsch et al. 1985) with selection on 100 μg/ml kanamycin. Media were as in Velten and Schell (1985).

Kanamycin-resistance seed assay. Tobacco seeds were aseptically removed from the capsules and plated on half strength Murashige and Skoog (1962) medium (MS/2) with 300 μg/ml kanamycin sulfate (Sigma), 1% sucrose and 3.5 g/l Gelrite agar. After five days in the dark, the plates were grown under 16 h/day light for three additional weeks.

DNA cloning and manipulations. Bacterial cells were made competent for DNA transformation by the methods of Hanahan (1983) or Maniatis et al. (1982). Bacterial plasmid DNA was prepared by the methods of Birnboim and Doly (1982) and large scale preparations were further purified by cesium chloride gradient ultracentrifugation as described in Maniatis et al. (1982). Extraction of *Agrobacterium* DNA was by the method of Dhaese et al. (1979). Restriction endonucleases and other DNA modification enzymes were purchased from Bethesda Research Labs, Boehringer Mannheim or Pharmacia and used as recommended by the suppliers. DNA was purified after each modification by two extractions with phenol-chloroform and precipitated by the addition of two volumes of ethanol in the presence of 0.3 *M* sodium acetate. Radioactive nucleotides were purchased from New England Nuclear and unlabeled nucleotides (deoxy and dideoxy) were purchased from Pharmacia. Acrylamide and agarose gel electrophoresis were performed with TBE buffer as described by Maniatis et al. (1982).

Plant nucleic acid extractions and hybridizations. Plant DNA and RNA were extracted by the methods of Taylor and Powell (1983) and Cashmore (1982), respectively. DNA was analyzed by agarose gel electrophoresis in 0.1 *M* NaCl, 0.089 *M* Tris, 0.089 *M* boric acid, 0.002 *M* EDTA (HTBE) buffer and in the presence of 0.5 μg/ml ethidium bromide. RNA was analyzed by formaldehyde agarose gel electrophoresis as previously described by Lehrach et al. (1977). Blotting was as described in Maniatis et al. (1982). Single stranded RNA probes for hybridizations were synthesized using the vectors pSP64 and pSP65 by the procedures of Melton (Green et al. 1983; Melton 1985; Melton et al. 1984). Hybridizations were performed in 45% formamide, 0.81 *M* NaCl, 45 m*M* sodium phosphate, pH 7.0, 4.5 m*M* EDTA, 100 μg/ml salmon sperm DNA, 150 μg/ml yeast tRNA, 10× Denhardt's solution and 0.2% SDS at 50° C and 62° C for DNA and RNA blots respectively. Filters were washed in three 30 min changes of 3 × SSC, 0.5% SDS then 0.3 × SSC, 0.5% SDS at the hybridization temperatures, wrapped moist in Saran wrap and autoradiographed with Kodak XAR5 film and two Dupont Cronex intensifying screens at −70° C.

DNA sequence analysis. Plasmids were sequenced using the dideoxy method of Sanger (Sanger and Coulson 1975, 1978; Sanger et al. 1977), with modifications for supercoiled dsDNA sequencing (Chen and Seeburgh 1985) using either the M13 15 bp universal primer (for pUC plasmids and M13 single stranded templates) or the SP6 polymerase promoter sequence primer (Promega Biotech). The reaction mixes were electrophoresed on 0.4 mm thick urea/polyacrylamide gels with 1 × TBE buffer (Sanger and Coulson 1978), and autoradiographed as described above.

Results

Construction of the chimeric beta-tubulin gene

The soybean beta-1 tubulin gene (SB-1) was isolated from a genomic library, cloned into bacteriophage lambda, and subcloned into pUC13 (designated pMG2SB1-8.1). Fig. 1 shows the construction of the chimeric tubulin gene and the intermediate vector used in these studies. In order to unambiguously distinguish the soybean beta-1-tubulin gene (SB-1) transcripts from the endogenous tobacco beta-tubulin mRNA, the soybean 3′ untranslated sequence was replaced with the non-homologous 3′ untranslated sequence of transcription unit 7 from the TL-region of the octopine Ti plasmid pGV117 (Dhaese et al. 1979; Velten and Schell 1985). Since gene 7 was deleted from the Ti plasmid used in this study (pGV3850, Zambryski et al. 1983), this construct allowed the gene 7 3′ untranslated sequence (designated g7pA) to be used as a specific probe to assess the integration and expression of the chimeric gene in genomic DNA and poly(A)$^+$ RNA from transformed tobacco plants.

A series of deletion clones approaching the SB-1 stop codon were obtained by the method of Poncz et al. (1982). A limited Bal31 nuclease digestion of *Pst*I cut 0.72 kb *Pst*I-*Bam*HI fragment from pMGSB1-8.1, (previously subcloned into M13) was filled in with Klenow and cut with *Bam*HI.

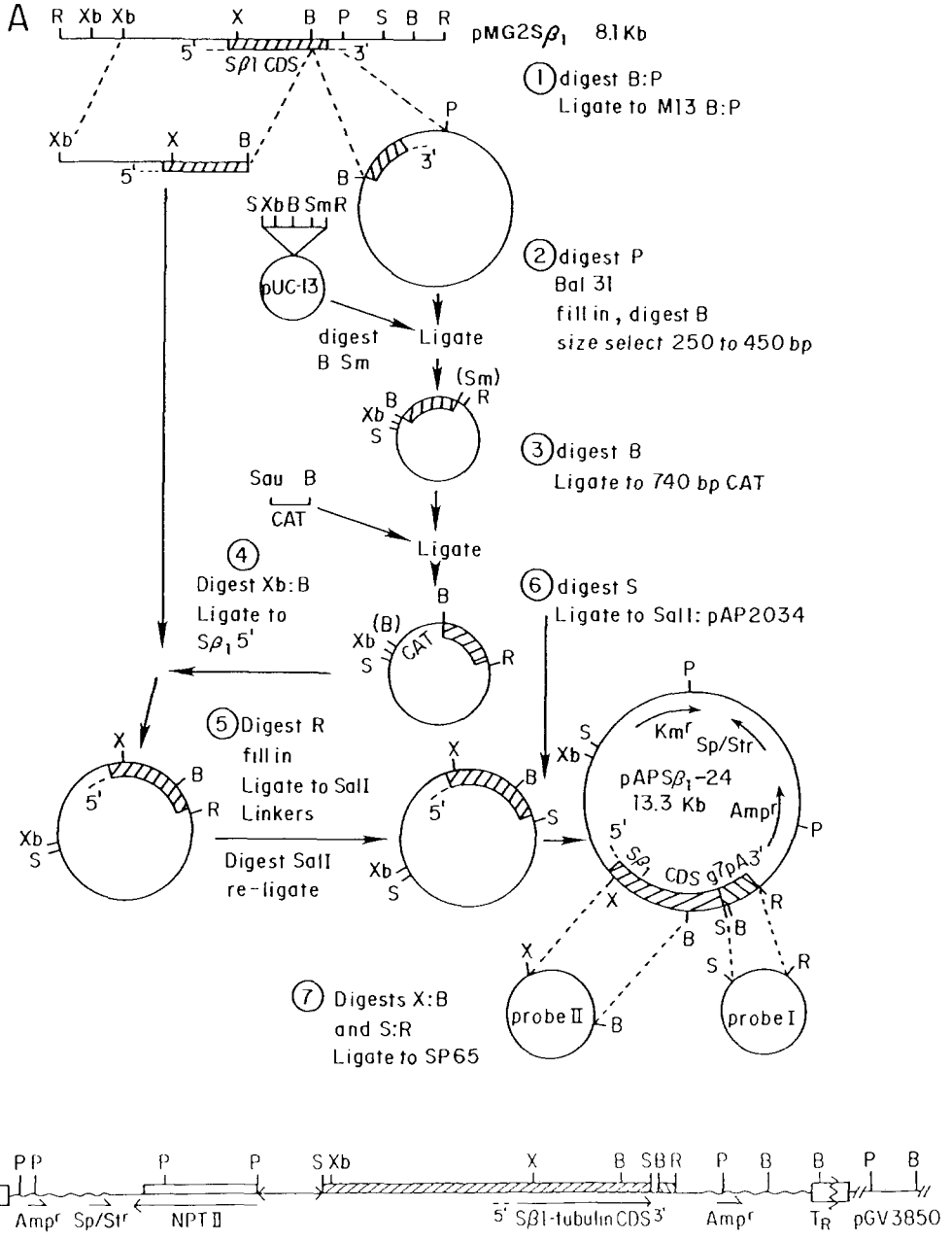

Fig. 1 A, B. Construction of chimeric beta-tubulin gene and structure of Ti plasmid cointegrate. **A** The 3′ end of the soybean beta-1-tubulin gene (indicated by *hatched boxes* and SB1-tubulin) was cloned into M13 and partially deleted with Bal31 nuclease, then recloned into pUC-13. After physical separation of the adjacent *Bam*HI and *Xba*I sites of the pUC polylinker, the SB-1 tubulin gene was reconstructed by the ligation of the 5′ coding sequences, putative promoter region and 2 kb of upstream soybean DNA sequences. After addition of *Sal*I linkers to the *Eco*RI site of the pUC polylinker, the construct was inserted into the Ti plasmid intermediate vector pAP2034 fusing the SB1 gene with the 3′ control region of gene 7 from the Ti plasmid (indicated by g7pA and *hatched boxes*). The origins of fragments used for antisense RNA hybridization probes using the vector SP65 are indicated. **B** Structure of the cointegrate Ti plasmid pGV3850::pAPSB124 after conjugation with *E. coli* and homologous recombination in *A. tumefaciens*. *Pst*I and *Bam*HI restriction sites are indicated. Abbreviations: P, *Pst*I; B, *Bam*HI; Xb, *Xba*I; S, *Sal*I; Sau, *Sau*3A; Sm, *Sma*I, R, *Eco*RI; {}, restriction sites which were destroyed by ligation of heterologous ends (i.e. *Sma*I to blunt ended Bal fragments, and *Sau*3A to *Bam*HI). CAT, chloramphenicol acetyltransferase gene, Km^r, kanamycin resistance for plant selection, Amp^r, Sp/St^r, ampicillin and spectinomycin/streptomycin resistance respectively for bacterial selection. T_L and T_R, left and right T-DNA borders respectively which are also indicated by upright *wavy lines*

Fragments between 200 and 450 bp were size fractionated on an acrylamide gel, recovered by electroelution into dialysis tubing, ligated to *Bam*HI-*Sma*I cut pUC13 and introduced into *E. coli* TB1 cells. Several of these clones were sequenced and one of these (pSB13′Bal24) was shown to be truncated after the first base of the SB1 stop codon (Fig. 2). The 4.0 kb *Xba*I-*Bam*HI fragment (isolated from *Bam*HI-*Xba*I-*Eco*RI cut plasmid pMG2SB18.1 as described above) was ligated to pSB13′Bal24 which had *Xba*I and *Bam*HI recessed ends derived from adjacent sites on the pUC13 polylinker sequence. Since the two sites are juxtaposed, to obtain excision at both sites, it was necessary to first physically separate them. This was accomplished by the insertion of a 220 bp *Bam*HI fragment containing

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SB1 3'      GCC ATG GCT GCA TGA GAA AAC CCT ... 1ØØ Nu.....
SB1 AA      ALA MET ALA ALA ***
Bal 24      GCC ATG GCT GCA T

           -13              -1{   pUC-13          }[ Sal I ]
Fusion 24   GCC ATG GCT GCA TGG GCG AGC TCG AAT TGG TCG ACG
Fusion AA   ALA MET ALA ALA TRP ALA SER SER ASN TRP SER THR

            24                                            59
Fusion 24   GAT CCC CCG ATG AGC TAA GCT AGC TAT ATC ATC AAT
Fusion AA   ASP PRO PRO MET SER ***

            6Ø                                            95
Fusion 24   TTA TGT ATT ACA CAT AAT ATC GCA CTC AGT CTT TCA
"       "   TCT ACG GCA ATG TAC CAG CTG ATA TAA TCA GTT ATT
"       "   GAA ATA TTT CTG AAT TTA AAC TTG CAT CAA TAA ATT
"       "   ATG TTT TTG CTT GGA CTA[>A+]TAA TAC CTG ACT TAT
"       "   TTT ATC AAT AAA TAT TTA AAC TAT ATT TCT TTC AAG
                                                      236
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Fig. 2. Nucleotide and predicted amino acid sequences of the soybean beta-1-tubulin gene 3′ end. The location of Bal deletion clone 24 (Bal 24) is shown as determined by the Sanger sequencing method, and the theoretical fusion junction (fusion 24) of the truncated beta-tubulin gene with the polyadenylation signal from gene 7 of the Ti plasmid (g7pA). Symbols: {} and [] indicate the pUC13 polylinker and *Sal*I linker sequences respectively; ***, stop codon; [>A+], poly(A) addition site. The two putative polyadenylation signals are *underlined*. Nucleotides are numbered relative to SB1 stop codon. (Sequences of the wild-type SB1 3′ and SB1 amino acids are shown at the top)

the Tn*9* gene chloramphenicol acetyl-transferase, (CAT) (Velten and Schell 1985), into the *Bam*HI site of the construct. Sequential cleavage of this plasmid with *Xba*I followed by *Bam*HI, released the CAT gene and produced the desired recessed ends for ligation. The resulting plasmid (pSB1tr24) contains the SB-1 tubulin gene including 2 kb of DNA 5′ of the transcriptional unit, but the gene is truncated at the translational stop codon (Fig. 2). The plasmid pSB1tr24 was linearized with *Eco*RI, filled in with Klenow, and ligated to *Sal*I linker fragments (Collaborative Research). The 4.3 kb *Sal*I fragment from this plasmid was ligated to *Sal*I cut pAP2034, fusing the SB-1 tubulin gene to the g7pA regulatory sequence and creating the final construction to be transferred to *Agrobacterium* (pAPSB124). The Ti plasmid intermediate vector pAP2034 includes a chimeric selectable marker gene for plants consisting of a bacterial neomycin phosphotransferase (NPT II) sequence under the control of plant active 5′ and 3′ regulatory sequences from the Ti plasmid (Velten et al. 1984). Transformed tobacco cells containing this gene were shown to contain active NPT II protein and to be resistant to 100 μg/ml kanamycin.

Figure 2 shows the nucleotide sequence of the wild-type SB-1 tubulin 3′ end, the location of Bal-31 deletion clone 24 and the theoretical sequence of the fusion junction of the chimeric gene. The Bal-31 deletion clone 24 is truncated just after the first nucleotide of the stop codon of the SB-1 coding sequence. Approximately 200 bp downstream from the fusion is the polyadenylation signal from gene 7 (g7pA).

Transformation of tobacco leaf disks

The construction and control vectors were transferred to *Agrobacterium tumefaciens* and integrated into the Ti plasmid pGV3850 (Zambryski et al. 1983) by the tri-parental transconjugation method (Van Haute et al. 1983). The structure of the cointegrate plasmid is shown in Figs. 1 and 3. The expected structures of the plasmids from several independent *A. tumefaciens* exconjugates were verified by restriction analysis and Southern blot hybridization to probe 1 (Fig. 3, lanes 1–4) and probe 2 (data not shown). Analysis of these data indicated that, although most of the plasmids had the predicted structures (5 out of 8), several showed minor deletions and insertions in the pBR322 DNA sequences (data not shown). This was probably the result of additional recombination events during the conjugation steps.

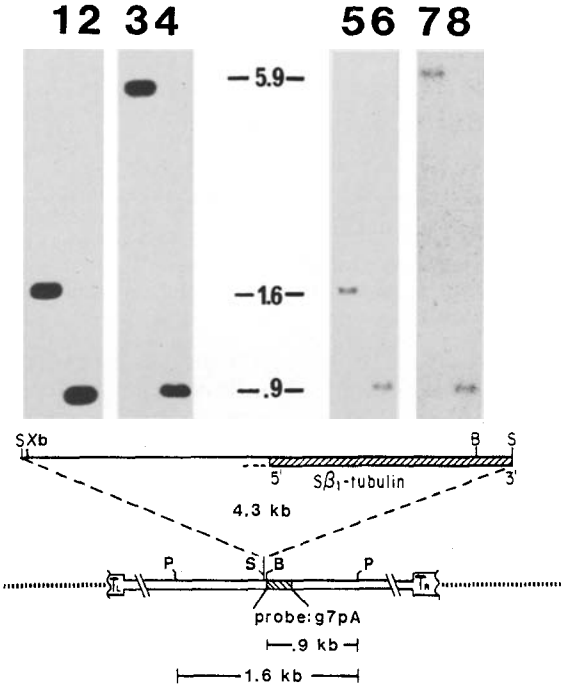

Fig. 3. Analysis of Ti plasmid cointegrate structures in *A. tumefaciens* and of the T-DNA insertions in tobacco genomic DNA by Southern blot hybridization analysis. Five micrograms of bacterial (lanes 1–4) and plant (lanes 5–8) DNAs were digested with *Pst*I alone (lanes 1, 3, 5, 7) and with *Pst*I and *Bam*HI (lanes 2, 4, 6, 8), run on agarose gels, blotted to nitrocellulose, hybridized to ^{32}P-labeled g7pA RNA probe 1 and washed as described in Materials and methods. Unmodified control plasmid (pGV3850::pAP2034) and DNA from plants transformed with this vector are in lanes 1–2 an 5–6 respectively. Plasmid with the chimeric beta-tubulin gene (pGV3850::pAPSB124) and corresponding tobacco genomic DNAs are in lanes 3–4 and 7–8 respectively. Autoradiography was for 16 h (lanes 1–4), 48 h (lanes 5–6) and 72 h (lanes 7–8). Fragment sizes in kb relative to molecular weight markers (not shown) are indicated. *Below* is a schematic drawing of the predicted structure of the cointegrate Ti plasmids and corresponding genomic insertions, with *dotted lines* indicating either pGV3850 or tobacco genomic DNA respectively. The origin of the g7pA probe, pertinent restriction enzyme sites and fragment sizes are indicated. Abbreviations are as in Fig. 1

Strains of *Agrobacterium* containing the recombinant Ti plasmids were used to transform leaf disks of *Nicotiana tabacum* Petit Havana cv. SR1 (Horsch et al. 1985). Selection with 100 μg/ml kanamycin yielded calli which formed

Table 1. Genetic analysis: segregation of kanamycin-resistance

Seed stock	Plated seeds	Germinated seeds	Resistant seedlings	Sensitive seedlings	Segregation ratio $Km^r:Km^s$ $p \leq 0.05$
A) Self fertilized transformants, S1 progeny					
Controls					
SR1[a]	222	164	0	164	0
T-AP-2A[b]	100	100	77 (75)	23 (25)	3:1
Experimental					
T-APSB124-1A	122	115	86 (86)	29 (28)	3:1
T-APSB124-1C	100	95	67 (71)	28 (24)	3:1
T-APSB124-2G	100	96	71 (72)	25 (24)	3:1
T-APSB126-1A	100	99	71 (74)	28 (25)	3:1
T-APSB124-1B	100	61	54 (54)	7 (7)	8:1
T-APSB124-1D	100	94	0	94	0
T-APSB124-1E	100	66	0	66	0
B) Self fertilized S1 progeny, S2 progeny					
T-APSB124-1A I	260	224	220 (224)	4 (0)	100%
T-APSB124-1A II	305	214	213 (214)	1 (0)	100%
T-APSB124-1A III	343	254	196 (190)	58 (64)	3:1
T-ABSB124-1A IV	266	221	164 (166)	57 (55)	3:1
T-APSB124-1A V	310	257	187 (193)	70 (64)	3:1

Expected numbers are in parentheses

[a] Untransformed, seed grown tobacco (SR1)
[b] Transformed with Ti plasmid not containing chimeric beta-tubulin gene

shoots over the course of six weeks. The shoots were excised and rooted in hormone-free MS/2 media containing 100 μg/ml kanamycin. After 2–4 weeks, plantlets with vigorous root growth were explanted into sterile soil and gradually acclimated to greenhouse conditions. These plants (R1), and all subsequent progeny examined (self cross progeny, S1 and S2), were morphologically identical to seed grown, untransformed control plants. From approximately 100 leaf disks transformed per construct, up to 250 independent transformants were observed. Thus the potential number of transformants plants far exceeded those actually recovered.

Segregational analysis

Regenerated plants (R1) were self fertilized either by placing them in indivdual rooms or by bagging the flowers before they opened. Bagging reduced the germination rates of the seed thus produced by about 25%. The segregation of kanamycin resistance among the progeny plants (S1) was assayed by plating seeds on MS/2 media containing 300 μg/ml kanamycin. The results are presented in Table 1 A. Seedlings were scored, after four weeks of growth, as kanamycin-resistant if they produced primary leaves or sensitive if they had germinated but did not form primary leaves. The cotyledons of sensitive plants were either yellow or white. The segregation ratios are expressed relative to the total number of germinated seeds because it was observed that the germination rates of the individual seed batches were similar on MS/2 media with or without kanamycin.

Data were analyzed by the chi square test ($p<0.05$) against the expected Mendelian segregation ratios. These results show that, of 8 transformants tested, 5 show a close fit to the expected Mendelian segregation ratio for a self cross with a single, dominant gene (3:1). One plant had an abnormal ratio (8:1) and 2 produced no resistant progeny.

Based on these results the R1 plants most likely to contain a single chromosomal insertion were chosen for subsequent analysis (T-APSB124-1A, containing the chimeric SB-1 gene and T-AP-2A, containing the unmodified transformation vector). Five kanamycin resistant S1 progeny from T-APSB124-1A were self pollinated and the seeds (S2) were assayed for kanamycin resistance (Table 1 B). The segregation ratios of three of these plants were as expected for a single heterozygous dominant gene (3:1). Two S1 plants (T-APSB124-1A I and II) produced 100% resistant S2 progeny as would be expected from homozygous individuals. A few progeny from these samples germinated but did not extend primary leaves. Abortive seedlings have been observed occasionally in both transformed and control SR1 tobacco growing on MS/2 without kanamycin at frequencies up to 4%. Therefore the appearance of several in the homozygous stocks derived from the R1 plant T-APSB124-1A is insignificant.

Molecular analysis

DNA and RNA were isolated from the leaves of individual transformed and control plants after the first seeds were harvested. DNA was digested with either *Pst*I alone or *Bam*HI and *Pst*I, then run on an agarose gel and blotted onto nitrocellulose. Poly(A)$^+$ RNA was fractionated on an oligo-dT cellulose column and samples were run on a formaldehyde agarose gel. The blots were hybridized to antisense RNA probe 1 or probe 2, made using SP6 polymerase (see Fig. 1). Probe 1 (g7pA) represents the 3′ untranslated region and polyadenylation signal of the fusion gene derived from a *Bam*HI-*Eco*RI digest of the plasmid pUA110 (Velten and Schell 1985). Probe 2 (SB1CDS) is the 1.4 kb

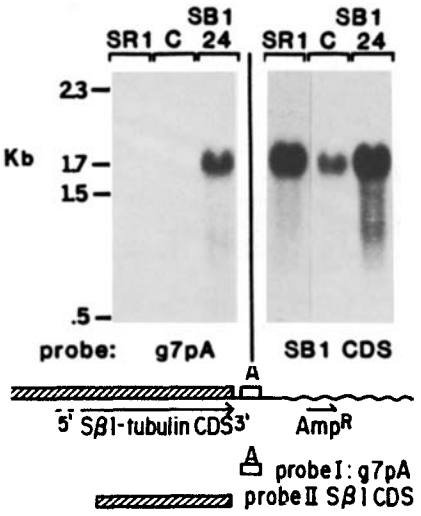

Fig. 4. Expression of the chimeric tubulin gene in transformed tobacco plants. Approximately 10 μg (lanes 1 and 4) and 5 μg (lanes 2, 3, 5 and 6) of poly(A)$^+$RNA was isolated, run on a agarose/formaldehyde gel, blotted to nitrocellulose and hybridized to ^{32}P-labeled RNA probes as described in Materials and methods. SR1 and C indicate RNA from untransformed and transformed control plants respectively and SB124 indicates RNA from plant T-APSB124 containing the chimeric tubulin gene. The *left panel* was probed with the g7pA sequence specific for the chimeric gene and the *right panel* was probed with the beta-tubulin coding sequence probe (SB1 CDS). The probes are indicated below the autoradiograms and in Fig. 1. Autoradiography was for either 3 days (probe 1) or 2 days (probe 2). The positions and fragment sizes in kb of end-labeled DNA molecular weight markers (not shown) are indicated. The size of the beta-tubulin mRNA (1.7 kb) relative to these markers is also indicated

*Xho*I-*Bam*HI fragment from pMG2SB18.1 containing most of the soybean beta-1-tubulin coding sequence. Both probes were cloned into the plasmid vectors SP65 and SP64.

The hybridization patterns produced with the transformed tobacco genomic DNA using the g7pA probe are identical to the pattern obtained from a Southern blot of Ti plasmid DNA (isolated from the *A. tumefaciens* strains used to transform the plants and cut with the same enzymes, Fig. 3).

These data demonstrate that, in these *Agrobacterium* strains and in individual transgenic plants, the *Pst*I and *Bam*HI fragments immediately flanking the g7pA sequence and the SB-1 tubulin gene have not been rearranged during the transconjugation and transformation events. The 4.3 kb *Pst*I fragment size difference between lanes 5 and 7 with DNAs from control and experimental plants confirm the presence of the SB-1 tubulin fragment in the plant (TpAPSB124-1A; lane 7) and its absence in the control plant (TpAP-2A; lane 5). The fragment sizes are in close agreement with the sizes predicted from the known maps. From these data it can be concluded that the wild-type tobacco genome contains no sequences that cross-hybridize to the g7pA probe at moderate stringencies.

Presented in Fig. 4 are autoradiograms from a Northern blot of RNA from untransformed (SR1), transformed control (C) and transgenic (SB124) tobacco plants hybridized to both probe 1 (g7pA, left panel) and probe 2 (SB1CDS, right panel). The soybean beta-1-tubulin coding sequence probe 2 detected beta-tubulin message in all of the samples, but the g7pA probe 1 sequence hybridized only to RNA from the plant carrying the chimeric gene (SB124). The chimeric mRNA transcripts in the transgenic plants are identical in size (1.7–1.9 kb) to the endogenous tobacco and soybean transcrips (probe 2, lane SR1).

Discussion

The data presented here demonstrate that the chimeric SB-1 tubulin gene and control vectors (pGV3850:pAP2034) were stably integrated into the genomes of the regenerated tobacco plants without any detected rearrangements (Fig. 3), and that proper sized beta-tubulin transcripts accumulate in the plant containing the SB-1 gene (Fig. 4). Lack of signal in untransformed (SR1) and transformed control RNA (C) when hybridized to the g7pA SP6 transcript (Fig. 4, probe 1) demonstrates the specificity of the probe, which does not cross hybridize with endogenous tobacco transcripts or to transcripts originating from the genes cointegrated with the Ti plasmid constructs. The hybridization of probe 1 to a 1.7 kb class of poly(A)$^+$RNA from a transgenic tobacco plant demonstrates that the chimeric SB-1 gene is transcribed in tobacco plants (Fig. 4, SB124, probe 1). Hybridization of control RNA to probe 2 (Fig. 4, right panel, SR1) demonstrates that tobacco tubulin transcripts will hybridize to the heterologous soybean beta-tubulin probe at the indicated stringencies indicating a high degree of conservation between the two species.

The presence of the 1′–2′ promoter fragment in the Ti plasmid intermediate vector (pAP2034) should not effect the level of transcription of the chimeric tubulin gene because sequence data indicate that the soybean DNA flanking the SB-1 gene contains short repetitive sequences which would terminate any read-through transcription. If this were not the case a shift in the molecular weight of the transcripts would have occurred. Even after prolonged exposure of the autoradiogram depicted in Fig. 4 no specific hybridization greater than 1.7 kb was seen. It can therefore be concluded that the cis-acting sequences of the soybean beta-1 tubulin gene responsible for expression are included within the 4.3 kb DNA fragment used in this study and that this putative promoter region is recognized by transcriptional factors in tobacco cells. The level of expression suggests a high degree of compatibility between the two species even though they diverged approximately 10 million years ago.

The fusion of the foreign 3′ regulatory sequences to the SB-1 gene 3′ end resulted in a polyadenylated RNA of the proper size containing a unique 3′ untranslated marker sequence useful as a gene specific probe. This demonstrates that the native 3′ untranslated sequence of the SB-1 tubulin gene can be replaced without disrupting efficient transcription of the gene, although the possibility that it may be involved in some other mode of gene regulation such as message stability or translatability can not yet be ruled out. Because the 3′ untranslated sequences of some tubulin genes have been conserved between species, it has been suggested that they are functionally important. This hypothesis is currently being tested by analyzing the patterns of regulation of the chimeric gene in tobacco, and comparing this with the expression of unmodified tubulin genes both in soybean and in tobacco.

The neomycin-phosphotransferase fusion gene included in the transformation vectors used for this study conferred

kanamycin resistance to SR1 tobacco cells and the majority of regenerated plants transmitted this phenotype stably through two generations as a single, dominant Mendelian trait. S1 plants homozygous at the loci were identified by analysis of the progeny of self crosses. However, the results presented in Table 1 show that one of the regenerated plants exhibited an abnormal segregation ratio (8:1). This might indicate that the leaf disk transformation method may occasionally result in plants with multiple inserts or of chimeric composition. This could be due to the close proximity of transformed and non-transformed cells in the leaf disks during regeneration.

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