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A functional analysis of T-DNA gene *6b*: The fine tuning of cytokinin effects on shoot development

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Summary. The physiological function *in planta* of T-DNA gene *6b* was studied under various experimental conditions. For this purpose the coding region of gene *6b* was cloned behind the 1'-promoter of the T_R-DNA to enhance expression of the gene product in transformed plant cells. Expression of the recombinant gene in leaf discs of *Nicotiana tabacum* altered the capacity for shoot formation of the discs, induced by exogenous (i.e. BAP in the growth medium or agrobacterial *trans*-zeatin produced under control of gene *tzs*) or endogenous cytokinins (i.e. isopentenyladenosine produced under control of T-DNA gene *4*). The data obtained indicate a reduction of cytokinin activity within the plant cells by the product of T-DNA gene *6b*.

Key words: *Agrobacterium tumefaciens* – Transformation – Leaf disc infection – *Nicotiana tabacum* – T_R promoter

Introduction

Agrobacterium tumefaciens causes so-called crown gall tumour, a neoplastic disease of many dicotyledonous plants. Tumorous growth is the consequence of transfer, stable integration and gene expression of a segment from a large bacterial Ti plasmid in the plant nuclear genome (T-DNA or transferred DNA; Zaenen et al. 1974; Chilton et al. 1977; Willmitzer et al. 1980). The T-DNA codes for several functions which are actively transcribed in tumour tissues. These genes are numbered according to the size of their transcripts, with two genes coding for transcripts of similar size named '*6a*' and '*6b*' (Willmitzer et al. 1983).

Some of the genes have been characterized functionally by transposon mutagenesis and in vitro expression of parts of the T-DNA. Thus, T-DNA gene *3* encodes either the octopine or nopaline synthase, responsible for the synthesis of opines – unusual amino acid derivatives produced by transformed plant cells (Tempe et al. 1979; Leemans et al. 1982; Joos et al. 1983). Genes *1, 2* and *4* encode enzymes for the biosynthesis of phytohormones, namely cytokinins and auxins. Barry and coworkers (1984) and other authors (Akiyoshi et al. 1984; Buchmann et al. 1985) have presented direct evidence that T-DNA gene *4* encodes the enzyme dimethyl-allylpyrophosphate:AMP transferase (DMA-transferase). The enzyme catalyses the covalent linkage of dimethylallylpyrophosphate to the N^6 of AMP, yielding isopentenyladenosine-5'-monophosphate, which is the first cytokinin in a complex series of metabolic pathways known for this class of phytohormones (see Letham et al. 1982).

Similarly, extensive in vivo and in vitro analysis of genes *1* and *2* supported the idea (Leemans et al. 1982; Joos et al. 1983) that these genes are involved in biosynthesis of the highly active auxin, indole-3-acetic acid (IAA). Indeed, T-DNA gene *1* encodes a monooxygenase which converts tryptophan to indole-3-acetamide (Van Onckelen et al. 1985, 1986; Thomashow et al. 1986), which is converted to IAA by a gene *2* encoded amidohydrolase (Inze et al. 1984; Schröder et al. 1984). The expression of genes *1, 2* and *4* from the T-DNA of Ti plasmids of *A. tumefaciens* therefore interferes with the normal hormonal regulation of plants, leading to elevated cytokinin and auxin levels in tumour tissues, as first observed by Weiler and Spanier (1981) and Akiyoshi et al. (1983).

Messens et al. (1985) postulated that gene *6a* is involved in the control of excretion of opines from the plant cell, wheras possible functions of genes *5* and *7* are not yet known (gene *7* is absent from Ti plasmids of the nopaline type; see Willmitzer et al. 1983). Observations by Leemans et al. (1983) and Ream et al. (1983) led to the notion that T-DNA gene *6b* is also involved in the regulation of phytohormone levels within tumour tissues. Leemans et al. (1983) postulated an anti-cytokinin effect for the *6b* gene product to explain the observed reduction of shoot formation in tumour tissues expressing both genes *4* and *6b*, compared to tissues expressing gene *4* only, whereas Ream et al. (1983) observed the reverse. No conclusion could therefore be drawn from these observations.

We designed experiments to demonstrate that the product of gene *6b* counteracts the effect of cytokinins with regard to shoot formation. Since T-DNA gene *6b* is poorly expressed in tumour tissues (Burrell et al. 1985) we constructed a chimeric gene with the coding region of gene *6b* under control of the strong 1'-T_R-promoter (Velten et al. 1984), hoping thus to enhance the biological effect produced by this gene. The phenotype resulting from the expression of this chimeric gene in transgenic tobacco tissues

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Abbreviations: AMP, adenosine-5'-monophosphate; BAP, 6-benzylaminopurine

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was studied using isolated leaf discs in the presence of different concentrations of cytokinins. This was accomplished either by adding BAP to the growth medium, or by expressing the agrobacterial *tzs* gene (Beaty et al. 1986) during the infection process or by co-expressing the cytokinin gene *4* in transgenic tobacco tissues.

Materials and methods

Bacteria and plasmids. All bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in YT medium (Miller 1972), supplemented with the appropriate antibiotics.

Cloning of T-DNA genes. Isolation, digestion (with restriction enzymes and exonuclease *Bal*31) and ligation of DNA was performed according to Maniatis et al. (1982). For producing blunt ends after restriction of DNA, the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer) or T4 DNA polymerase (New England Biolabs) were used for 5′- and 3′-protruding ends, respectively. The *Bgl*II-linker [d-(CAGATCTG), New England Biolabs] was phosphorylated according to Chaconas et al. (1975).

Transfer of plasmids to Agrobacterium. The conjugational transfer of recombinant plasmids to *A. tumefaciens* was performed according to Van Haute et al. (1983) with the *E. coli* helper strain GJ23. Transconjugants were selected on

Table 1. Bacteria, plasmids and phages used

	Description	Reference
Agrobacterium strains:		
C58C1	C58 derivative without Ti plasmid	Van Larebeke et al. 1974
C58C1 (pGV2260)	Octopine type Ti plasmid; T_L- and T_R-DNA replaced by pBR322	Deblaere et al. 1985
C58C1 (pGV3850)	Nopaline type Ti plasmid; T-DNA with the exception of genes *3* and *a* replaced by pBR322	Zambryski et al. 1983
C58C1 (pGV2260, pBTR6)	T-DNA gene *6b* with 1′-promoter	This work
C58C1 (pGV2260, pBTR46)	T-DNA gene *6b* with 1′-promoter in combination with T-DNA gene *4*	This work
C58C1 (pGC2260, pBVG6)	T-DNA gene *6b* with its own promoter	This work
C58C1 (pGV2260, pBVG46)	T-DNA gene *6b* with its own promoter in combination with T-DNA gene *4*	This work
C58C1 (pGV2260, pBVK6)	T-DNA gene *6b* without promoter	This work
C58C1 (pGV2260, pBVK46)	T-DNA gene *6b* without promoter in combination with T-DNA gene *4*	This work
C58C1 (pGV2260, pBVG4)	T-DNA gene *4*	This work
C58C1 (pGV2260, pBVG4neo)	T-DNA gene *4* in combination with gene *npt*II	This work
C58C1 (pGV3850::pSTR1)	T-DNA gene *6b* with 1′-promoter in combination with gene *tzs*	This work
Escherichia coli strains:		
BMH71-18	*Δ-(lac,pro)*, F′ *lacI*^{q}*Z Δ*-M15 *pro*$^+$	Messing et al. 1977
HB101	*Pro*$^-$ *Leu*$^-$ *His*$^-$ *lacY* r_R^- m_R^-	Boyer and Roulland-Dussoix 1969
GJ23	Helper strain for conjugative transfer; plasmids R64drd11 (*tra*), pGJ28 (*mob*)	Van Haute et al. 1983
E. coli plasmids:		
pUC8, pUC18, pUC19	pBR322-derivative with α-fragment of the *E. coli lacZ* gene and polylinker sequences	Vieira and Messing 1982
pHD103	*Bam*HI fragment 17a of pTiAch5 in pUR2-Bam	Rüther 1980; J. Schröder, personal communication
pGV0507	*Eco*Ri fragment 7 of pTiAch5 in pBR325	R. Deblaere, personal communication
pAS2022	1′-2′-dual promoter fragment of the T_R-DNA of pTiAch5 in pBR322; *npt*II gene of Tn*5* downstream of 2′-promoter	Velten and Schell 1985
E. coli plasmids:		
pGV943	pGV815 derivative with left and right T-DNA border sequences and wide host-range replication origin or *Pseudomonas* plasmid pVS1	Deblaere et al. 1985; Itoh et al. 1984; R. Deblaere, personal communication
pGV941	Derivative of pGV943 with *npt*II gene of pKC7::nos	Deblaere et al. 1985; R. Deblaere, personal communication
pSRB3	Derivative of pGV943 with 439 bp $lacZ_{alpha}$-fragment of pUC19 cloned between T-DNA borders	This work
Bacteriophages:		
M13mp9	M13 derivative with α-fragment of the *E. coli lacZ* gene and polylinker sequence	Vieira and Messing 1982

minimal agar plates, supplemented with octopine or nopaline and the appropriate antibiotics (BTB medium; Hooykaas et al. 1979).

Leaf disc transformation. Leaf discs of 8-week-old, aseptically grown plants of *Nicotiana tabacum* var. Petit Havanna "SR1" (Maliga et al. 1973) were inoculated with *Agrobacterium* on MS medium according to the method of Horsch et al. (1985). Co-infections of leaf discs with two bacterial strains were performed with a 1:1 mixture of the bacteria. Cultivation of plant material was performed in a growth chamber at 22°–25° C with 3000–5000 lux for a day length of 16 h. Two days after inoculation leaf discs were transferred to fresh MS medium, supplemented with the antibiotic claforan (Hoechst, 500 mg/l). Shoot formation was recorded qualitatively for 6–8 weeks, starting about 1 week after inoculation of the leaf discs.

Results

Sub-cloning of T-DNA genes 6b *and* 4

The coding region of T-DNA gene *6b* was derived from the *E. coli* plasmid pHD103 (Table 1). This plasmid contains the 4.8 kb *Bam*HI-fragment 17a of the octopine type Ti plasmid pTiAch5 with the genes *6a, 6b, 3* and part of gene *4* (Fig. 1; Willmitzer et al. 1983). From this plasmid a 1.8 kb *Bcl*I/*Pvu*II fragment with the coding region of gene *6b* was cloned into the *Bam*HI and *Sma*I site of pUC8. After linearization of the resulting plasmid with the enzyme *Eco*RI, cutting 5′ to the coding region of gene *6b*, the DNA was trimmed back with exonuclease *Bal*31. DNA molecules 250–300 bp shorter than the starting material were recircularized in the presence of a 70-fold excess of a *Bgl*II linker. Ligation of the *Bgl*II linker (5′-CAGATCTG) potentially creates a new recognition site for the restriction endonuclease *Pst*I (5′-CTGCAG) at each 5′-CAG trinucleotide at the 5′ end of the *Bal*31-digested DNA. Trinucleotides with the sequence 5′-CAG occur at 5′ positions 26, 32 and 41 bp, and 3′ positions 15, 72 and 120 bp relative to the codon ATG which is responsible for initiation of translation of gene *6b* (Fig. 2).

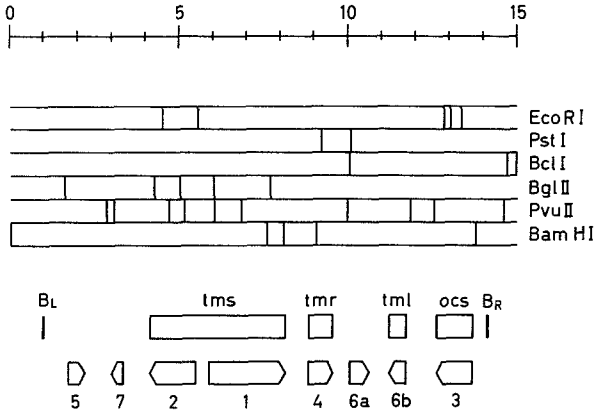

Fig. 1. T-DNA of an octopine type Ti plasmid. The length and direction of functional genes are indicated by *arrows*. Genetic loci, as characterized phenotypically by transposon mutagenesis, are indicated as *boxed areas*. The scale of the restriction map is in kb

The plasmid (pKSP3) with the *Pst*I site 32 bp upstream of the ATG (as confirmed by sequencing, data not shown) was chosen for further cloning of gene *6b* as a *Pst*I restriction fragment into pUC18, resulting in plasmids pKSK3 and pKSK4. These plasmids contained the coding region of gene *6b* in both orientations, each with multiple cloning sites available 5′ to the ATG (Fig. 3). Subsequently, with the construction of plasmid pSTR1 the coding region of gene *6b* was positioned behind the 1′-promoter of the T_R-DNA by introducing a *Hin*dIII/*Sal*I restriction fragment from pKSK3 into plasmid pAS2022 (Table 1, Fig. 4a).

For control experiments a restriction fragment of plasmid pHD103 containing the complete gene *6b* was introduced into pUC18, resulting in plasmid pORG6. Furthermore, T-DNA gene *4* (derived from plasmid pGV0507; Table 1) was introduced into plasmids pKSK4 and pORG6, leading to plasmids pKSK44 and pORG46, respectively.

To transfer the cloned genes into plants, plasmids pSTR1, pORG6, pORG46, pKSK4 and pKSK44 were used to construct a series of plasmids with various combinations of genes *6b* and *4* in the binary T-DNA vectors pSRB3 or pSRB4, respectively (Table 1; see Figs.4a, b for details and nomenclature of the resulting constructs). By conjugational transfer according to the method of Van Haute et al. (1983) the resulting plasmids were introduced into an *Agro-*

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5'-CAGCTGAAAA TTCAAACGCG CTAGTCAATG CTATCAATCT GTCGTGTTCA
   CACGGCATCA AACGGTCACC AATGACGTCA ATGGGCTTCC TTAGTCAATA
   TAAAAACCAA CGGCTCAGAC TTACCAGCGG CAGGTATTTG TAGTACATCC
   AACACTGCG

   ATG ACG GTA GCT AAT TGG CAG GTT CGA GAT TTG ACG CTT ATC
   CTG CGC ACC GGC GAG ATG AAG AGT CGC TTG GAA CAG GCG AGA
   ACG GAT TTC GGA GCG TTA CTG TCC GAA ACT GTA TAC TTT CAG
   CCT TCC GCG ATT AGA CTT GGT GAG TTT GAT GAC GAG TAC ATC
   CAC TCT CGA CAA GAG CTG GTC TAT GTA TAC CTT CGT GAA GAT
   ATT GCA CGC CAA TGC GCC TTG CGT CGA AAC CTA CCG TCC AAC
   TCC TCT AAC TTC GGA ACA ATG GCA ACT GCA ATA CCG CCG TGG
   CTG ATG AAT GCA CGC AGC CTG AAT CGA GTT ATG CAG GAA AGG
   TGC GAT CAA GGT GGC CTC GTC AAC TAC TAT CAA GGC CCA CAT
   ACA AAT CAG TTC TTT TTG GCG ATT ATG CCA AGC AAC TGC TTT
   GTT CGG TTC GGG ACC GAC ATA ATC AAC AAT GAA AAC TAC GGT
   TTT AAG CCC GGG GAG GAA TAC ACT AGA GGA AGG AGA AGA

   TGACGACGAT GAGATGGACG ATGAAGGGGA GGCTGGTGGA GCGGAACCAA
   GAGAGTGTCA GATCGGAAAC CTTATCAATT ATCCGATCAT TGCTTTAGGG
   TCATGCGATC TTTCCGCATA ATTCCCGTCG CCGACACCTA ATAAAGTCGG
   CTAATCTATG TGATTGAGTG TGTCTTGACT TTGTTATTTT GCATGTTTCC
   AATGTCATTT AGTAACGAAA TAAACGTTAT CCTCTTCTAA AAGCAGGCTG
   TGTTTTCGGC AAACATCGCC ACCCATCGCT AGTTTTTCTA AAAGTGTTCT-3'
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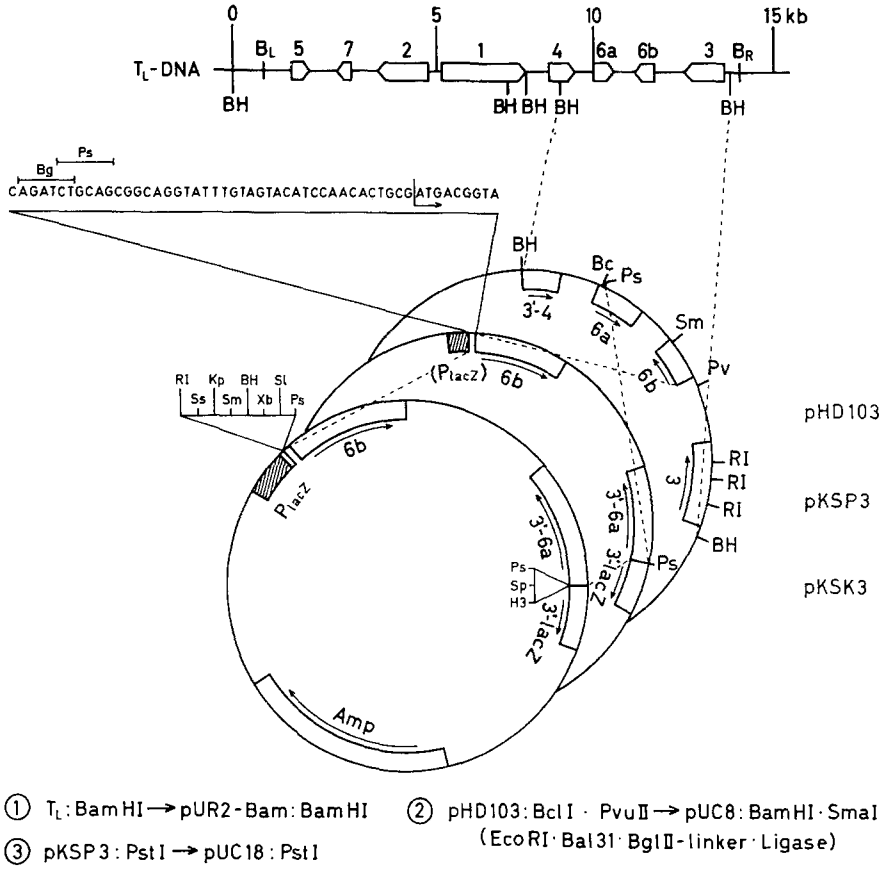
Fig. 2. Nucleotide sequence of T-DNA gene *6b* of plasmid pTiAch5 (Gielen et al. 1984). The coding region is listed as triplets. In the 5′-leading region common promoter elements are typed in *bold* face; in the 3′ region a possible polyadenylation signal is also indicated in *bold* face. 5′-CAG triplets in the vicinity of the ATG initiation codon of the gene are *underlined*; the CAG used for gene fusion is *boxed* (see the Results)



① T_L: BamHI → pUR2-Bam: BamHI ② pHD103: BclI · PvuII → pUC8: BamHI · SmaI (EcoRI · Bal31 · BglII-linker · Ligase)

③ pKSP3: PstI → pUC18: PstI

Fig. 3. Cloning steps of the promoter-less gene *6b* construct. Starting from pHD103 a fragment containing the complete T-DNA gene was recloned into pUC8. The promoter region of up to 32 bp 5′ to the initiating ATG was deleted by *Bal*31 digestion. After ligation to a *Bgl*II linker a *Pst*I fragment was recloned into pUC18, leading to pKSK3. The nucleotide sequence is as revealed by DNA sequencing. Abbreviations of restriction enzyme sites: BH, *Bam*HI; Bc, *Bcl*I; Ps, *Pst*I; Sm, *Sma*I; Pv, *Pvu*II; RI, *Eco*RI

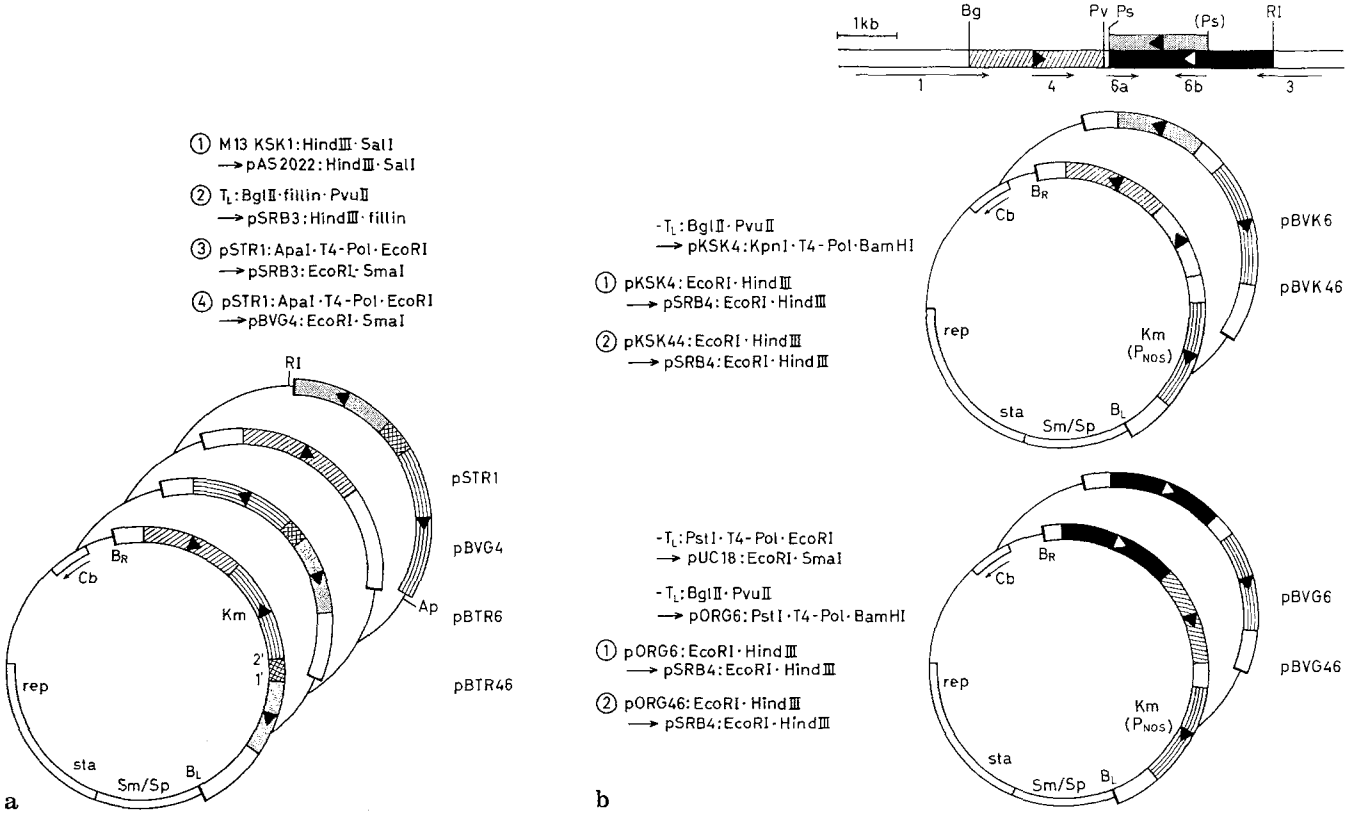


Fig. 4a and b. T-DNA constructs for functional analysis of the T-DNA gene *6b*. Different fragments of the T-DNA of pTiAch5 containing gene *6b*, with or without a functional promoter, and/or gene *4* were cloned into the binary vector plasmids pSRB3 or pSRB4. The promoter-less gene *6b* is indicated by the *dotted* area and gene *6b* with the wild-type promoter by the *black* area; *dashed boxes* indicate gene *4* (for extension of the cloned fragments see the T-DNA map in **b**). *Longitudinally hatched* boxes indicate the *npt*II gene. The direction of transcription of each gene is indicated by a *triangle*. rep, sta = wide host range replication origin and region of stability of the *Pseudomonas* plasmid pVS1; B_L, B_R = left and right T-DNA borders. **a** Constructs with gene *6b* under control of the 1′-promoter of the T_R-DNA (*double hatched boxes*). **b** Constructs of gene *6b* with the wild-type promoter or without promoter. Antibiotic markers are defined as: Sm/Sp = Streptomycin/Spectinomycin; Cb = Carbenicillin; Km = Kanamycin

bacterium strain containing the T-DNA-less, octopine type Ti plasmid pGV2260. Additionally, plasmid pSTR1 was transferred into an *Agrobacterium* strain containing the nopaline type Ti plasmid pGV3850 (Zambryski et al. 1983). The *Agrobacterium* strains used are listed in Table 1.

Functional characterization of gene 6b *by leaf disc infection*

The development of leaf discs of *Nicotiana tabacum* under the influence of different concentrations of the synthetic cytokinin, 6-benzylaminopurine (BAP), in the growth medium was studied in the absence of *Agrobacterium*. The earliest shoots appeared at day 18 after starting the experiment on medium with 0.5 mg/l BAP, whereas on media with 0.1 or 1 mg/l BAP shoots appeared later at days 22 to 24. However, no difference in the extent of shoot production could be observed after 30–40 days. Further increases of the cytokinin concentration to 2 or 5 mg/l BAP resulted in a strong inhibition or prevention of shoot formation. At 2 mg/l BAP occasional buds were formed, which mostly did not develop further. Based on these observations, leaf disc infections with the *Agrobacterium* mutants described above were performed on media containing the relevant BAP concentrations, as listed in Table 2.

Inoculation of leaf discs with *Agrobacterium* strains either devoid of any Ti plasmid or harbouring the disarmed octopine type Ti plasmid pGV2260 (Nos. 2 and 3 in Table 2) did not affect shoot production of the plant material as compared to uninfected leaf discs. In contrast, a clear difference in the morphogenic capacity of the discs was observed after inoculation with bacteria containing the disarmed nopaline type Ti plasmid pGV3850 (No. 4 in Table 2), harbouring gene *tzs* (*trans*-zeatin synthase) in its Vir region. On hormone-free medium a strong shoot proliferation appeared 10–12 days after starting the experiment, whereas addition of BAP to the growth medium reduced shoot production with increasing BAP concentrations.

Leaf discs infected with bacteria containing T-DNA gene *4* alone (Nos. 5 and 6 in Table 2) expressed strong shoot production after 32 days on hormone-free medium. On media containing BAP, shoot production induced by exogenous cytokinins was slightly reduced in the presence of T-DNA gene *4*; however, buds that were not able to develop (as observed at 2 mg/l BAP in the absence of gene *4*) appeared at 0.5 mg/l BAP. Likewise, *tzs*-induced shoot formation was slightly reduced in the presence of T-DNA gene *4* (No. 7 in Table 2).

Agrobacterium strains containing the promoter-less T-DNA gene *6b* construct were not able to alter the phenotypic reactions of the leaf discs under all conditions tested (Nos. 8–11 in Table 2). Furthermore, on hormone-free medium no reaction was observed when leaf discs were infected with bacteria containing a functional T-DNA gene *6b*, either under control of its own or the 1′-promoter of the T_R-DNA (Nos. 12 and 16 in Table 2). Leaf discs turned brown and degenerated after 3–4 weeks.

However, depending on the concentration of the phytohormones, a functional T-DNA gene *6b* under control of its own or the T_R-1′-promoter (Nos. 12–22 in Table 2) had a significant influence on the morphogenic capacity of the leaf discs when cytokinins were applied exogenously (either as BAP or as the result of the expression of gene *tzs*) or endogenously (as a result of the transfer and expression of T-DNA gene *4*). At a concentration of 0.5 mg/l BAP, or after co-infection on hormone-free medium with *Agrobacterium* containing either T-DNA gene *4* or gene *tzs*, the expression of gene *6b* significantly *reduced* shoot formation. Under super-optimal high concentrations of cytokinins, however, the expression of gene *6b stimulated* shoot production. Thus, the expression of gene *6b* allowed optimal shoot formation either on a medium containing 2 mg/l BAP, or when leaf discs were simultaneously inoculated with *Agrobacterium* expressing gene *tzs* on a medium containing 0.5 mg/l BAP. The respective phenotypes were more prominent if T-DNA gene *6b* was under control of the 1′-promoter of the T_R-DNA rather than under control of the wild-type promoter.

The activity of gene *6b* was analysed by measuring RNA

Table 2. Inoculation of *Nicotiana tabacum* leaf discs with strains of *Agrobacterium* on media with different BAP concentrations. Nos. 7, 9, 10, 13, 14, 17, 18, 20 and 22 are co-infections with two bacterial strains

No.	Strains used for infection	BAP concentration of the growth medium (mg/l)					
		0	0.1	0.5	1	2	5
1	no bacteria	+	+	+	+	+	+
2	C58C1	+	−	+	−	−	−
3	C58C1 (pGV2260)	+	−	+	−	−	−
4	C58C1 (pGV3850)	+	+	+	+	+	+
5	C58C1 (pGV2260, pBVG4)	+	+	+	+	+	+
6	C58C1 (pGV2260, pBVG4neo)	+	−	+	−	−	−
7	C58C1 (pGV3850)/ C58C1 (pGV2260, pBVG4)	+	−	+	−	−	−
8	C58C1 (pGV2260, pBVK6)	+	−	+	−	−	−
9	C58C1 (pGV2260, pBVK6)/ C58C1 (pGV2260, pBVGG4)	+	−	+	−	−	−
10	C58C1 (pGV2260, pBVK6)/ C58C1 (pGV3850)	+	−	+	−	−	−
11	C58C1 (pGV2260, pBVK46)	+	−	+	−	−	−
12	C58C1 (pGV2260, pBVG6)	+	−	+	−	−	−
13	C58C1 (pGV2260, pBVG6)/ C58C1 (pGV2260, pBVG4)	+	−	+	−	−	−
14	C58C1 (pGV2260, pBVG6)/ C58C1 (pGV3850)	+	−	+	−	−	−
15	C58C1 (pGV2260, pBVG46)	+	−	+	−	−	−
16	C58C1 (pGV2260, pBTR6)	+	+	+	+	+	+
17	C58C1 (pGV2260, pBTR6)/ C58C1 (pGV2260, pBTR4)	+	−	+	−	−	−
18	C58C1 (pGV2260, pBTR6)/ C58C1 (pGV3850)	+	−	+	−	−	−
19	C58C1 (pGV2260, pBTR46)	+	+	+	+	+	+
20	C58C1 (pGV2260, pBTR46)/ C58C1 (pGV3850)	+	−	+	−	−	−
21	C58C1 (pGV3850::pSTR1)	+	+	+	+	+	+
22	C58C1 (pGV3850::pSTR1)/ C58C1 (pGV2260, pBVG4)	+	−	+	−	−	−

+, Bacteria tested on the given BAP concentration; −, bacteria not tested on the given BAP concentration

isolated from transgenic plants containing gene *6b* under the control of its own or the 1'-promoter of the T_R-DNA. In Northern type experiments mRNA was detected in roots of a transgenic plant expressing gene *6b* under the control of the 1'-promoter. The intensity of hybridization with leaf mRNA was very weak. A modified polymerase chain reaction (PCR) clearly detected gene *6b*-specific mRNA in leaves, at a level more than 10-fold less than in roots. No mRNA could be detected in leaves or in roots from a transgenic plant containing gene *6b* under control of its own promoter (Fritz 1988). The data of the leaf disc experiments are summarized in Table 3.

Discussion

The function of four of the "common" T_L-DNA genes of *A. tumefaciens* are known. T-DNA genes *1, 2* and *4* encode enzymes for auxin and cytokinin biosynthesis in transformed plant cells, and gene *3* encodes an enzyme that catalyses the synthesis of opines. Three types of experiments were previously performed to identify the function of these genes. Firstly, individual genes were inactivated by transposon mutagenesis and mutants were tested in vivo. The comparison of the tissue phenotypes obtained after inoculation with such mutants and the wild-type bacteria allowed some conclusions about the functions of the mutated genes (Garfinkel et al. 1981; Leemans et al. 1982; Joos et al. 1983). Secondly, T-DNAs were constructed which were deficient in all tumour genes except the gene of interest. Mutant strains containing these T-DNAs were used for inoculation of plant material and the resulting phenotypes were used to support the functional analysis (Inze et al. 1984). Thirdly, individual T-DNA genes were cloned into suitable vectors of *E. coli*, expressed in vitro and the gene products were tested enzymatically (Barry et al. 1984; Schröder et al. 1984; Buchmann et al. 1985; Gafni and Chilton 1985; Thomashow et al. 1986).

The analysis described here was designed to determine the function of T-DNA gene *6b*. Since experiments with T-DNA mutants obtained after transposon mutagenesis yielded no or contradictory results (Garfinkel et al. 1981; Leemans et al. 1983; Ream et al. 1983), the second strategy of expressing gene *6b* in a well defined mutant T-DNA was chosen. This strategy was crucial for the subsequent analysis of the function of T-DNA gene *6b*. Replacement of it's own promoter for the 1'-promoter of T_R-DNA (Velten and Schell 1985) in a transcriptional fusion resulted in a significantly higher expression of gene *6b*.

Leaf disc infection assays (Horsch et al. 1985) were used since leaf discs are very sensitive to changes in cytokinin levels. Treatment of the leaf discs with the synthetic cytokinin BAP in the absence of bacteria induced strongest shoot production at a concentration of 0.5 mg/l. Lower or higher cytokinin concentrations resulted in decreased shoot formation. This demonstrates that shoot formation of the leaf discs is regulated by the cytokinin BAP within a narrow range of concentrations.

Consistent with the observed sensitivity of the leaf discs towards BAP was the strong response to inoculation with an *Agrobacterium* strain harbouring the nopaline type Ti plasmid pGV3850 (Zambryski et al. 1983). Bacteria containing nopaline type Ti plasmids produce and excrete the cytokinin, *trans*-zeatin. The producton of this cytokinin is under control of the *tzs* gene, located in the Vir region of the Ti plasmid (Akiyoshi et al. 1985; Beaty et al. 1986). In the leaf disc system, the concentration and accessibility of zeatin produced by the bacteria are optimal for shoot formation. Since *tzs*-controlled shoot formation is not observed in whole plant inoculations with bacteria harbouring Ti plasmid pGV3850 (Zambryski et al. 1983), it must be concluded that isolated leaf discs are more sensitive to *trans*-zeatin than whole plants. Furthermore, this result demonstrates that the formation of shoot primordia might not be dependent on a prolonged and significant increase in cytokinin levels, since the cytokinin-producing bacteria were killed by the antibiotic claforan 2 days after infection. The high levels of cytokinins previously observed in shoot-producing tumour tissues were measured after the shooty phenotype was already established (Akiyoshi et al. 1983; Spanier 1987), whereas no increase in phytohormone levels compared to those of control tissues could be detected in early developmental stages (Spanier 1987). Therefore, analysing cytokinin levels within leaf discs inoculated by the various agrobacterial strains described here did not seem to be appropriate, since differences important to shoot differentiation were expected to be too small and restricted to a very few cells.

Inoculation of leaf discs with *Agrobacterium* strains con-

Table 3. Influence of T-DNA gene *6b* on the differentiation of *Nicotiana tabacum* leaf discs under different experimental conditions. Shoot production was induced by cytokinins from various sources, first signs appearing after times as indicated

	pGV3850 gene *tzs*	BAP in mg/l			T-DNA gene *4*	0.5 mg/l BAP	
		0.5	1/0.1	2		+ gene *tzs*	+ gene *4*
Without gene *6b*:							
Shoot production	+++	+++	+++	(+)	+++	+	++
After days	10–12	18	21–24	44	30	10–12	19
With gene *6b*:							
Shoot production	++	++	+	++	++	++	+++
After days	11–13	19	23–26	42	33	10–11	18

Gene *tzs*, "zeatin" gene in the Vir region of nopaline type Ti plasmids; BAP, synthetic cytokinin in the growth medium; gene *4*, "cytokinin" gene of the T-DNA

The capacity for shoot production varied gradually (+++, very strong; ++, intermediate; +, weak; (+), shoots eventually after long time

taining T-DNA gene *4* resulted in a slight reduction of BAP or gene tzs-induced shoot formation. This suggests that the endogenous presence of the cytokinin isopentenyladenosine-5′-monophosphate can be monitored by the test system. However, the effect is delayed, since induction of gene *4* is dependent upon transfer and integration of the T-DNA gene into the plant genome.

The sensitivity of isolated leaf discs towards different concentrations of cytokinins and the resulting response of the plant material by shoot formation, allowed us to study unambiguously the influence of T-DNA gene *6b* on concentration dependent cytokinin activity in vivo. All experiments consistently demonstrated that T-DNA gene *6b* influences the shoot formation capacity of tobacco leaf discs, probably via an effect on the activity of functional cytokinins within the plant material. The data summarized in Table 3 suggest that the expression of gene *6b* reduces the activity of biologically active cytokinins, irrespective of the type of cytokinin (i.e. BAP, *trans*-zeatin or isopentenyladenosine-5′-monophosphate) and both for exogenously supplied or endogenously produced cytokinins. Such a reduction of cytokinin activity could be explained by influence controlled by gene *6b* on either the concentration of the plant hormone itself, the susceptibility of the plant material towards the hormone or stimulation of the antagonistic effect of another plant hormone, like auxin. Since a variety of obviously inactive derivatization and degradation products are known for cytokinins (Letham et al. 1982), it is conceivable that the gene *6b* product is involved in a derivatization or degradation pathway leading to reduced levels of biologically active cytokinins. On the other hand, a reduction of the plant material's ability to respond to the phytohormone by either blocking a specialized transport system or reducing the sensitivity or number of receptor molecules could also explain our results.

Finally, auxins act as antagonists to cytokinins in many plant systems (Scott 1984), so that a stimulation of auxin effects by the gene *6b* product through production of this phytohormone or increased sensitivity of the auxin response is also possible. The experiments described here are not able to discriminate between these possibilities. However, expression of the gene product in an in vitro system and analysis of the effects on different cytokinins are in progress, which might aid further understanding of the role of T-DNA gene *6b*.

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