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Exogenous Phytohormone-Independent Growth and Regeneration of Tobacco Plants Transgenic for the *6b* Gene of *Agrobacterium tumefaciens* AKE10[1]

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The *6b* gene of *Agrobacterium tumefaciens* AKE10 (AK-*6b*) induces crown gall tumors on certain plants but so far there have been no reports of the gene being able to induce tumors on culture medium. We cloned T-DNA segments containing the *6b* gene but lacking the auxin and cytokinin biosynthesis genes from *A. tumefaciens* AKE10. Tobacco (*Nicotiana tabacum*) leaf discs infected with *A. tumefaciens* LBA4404 carrying the clones produced shooty calli on hormone-free Murashige-Skoog medium. The relevant T-DNA segment was integrated into plant DNA as determined by Southern hybridization. Some of these immature shoots spontaneously developed into mature shoots, of which several leaves displayed morphological abnormalities. When leaf discs of these mature plants were placed onto the same medium numerous shoots developed from the wounding sites, indicating that the transgenic plants possessed a high regenerative potential. Northern blot and reverse transcriptase-polymerase chain reaction analyses showed a large accumulation of the AK-*6b* transcripts in the shooty calli, but only a limited degree in mature plants, demonstrating that AK-*6b* expression is regulated in plants and essential for the early stages of regeneration. Cytokinin levels in the shooty calli were comparable to those in normal shoots, suggesting that shoot regeneration is not mediated by the modulation of cytokinin content.

Tumorous crown gall disease of many plants is caused by infection with the soil bacterium *Agrobacterium tumefaciens*. During tumorigenesis T-DNA on the Ti plasmid harbored by the bacteria is transferred and integrated into host plant chromosomes (Van Larebeke et al., 1974; Chilton et al., 1977, 1980). Transformed tumorous tissues produce unique compounds, called opines, which include nopaline (Goldman et al., 1969), octopine (Ménage and Morel, 1964), and others (reviewed by Nester et al., 1984). Since the opine synthesis genes are located on the T-DNA, the Ti plasmids have been classified based on the type of opine they contain.

The auxin biosynthesis genes (gene *1* and gene 2) and the cytokinin biosynthesis gene (gene 4) are also located on the T-DNA, and expression of these oncogenes is required for production of the phytohormones, which ensure neoplastic growth of plant tissues (Akiyoshi et al., 1984; Thomashow et al., 1984). Thus, transformed plant cells can grow in in vitro culture medium in the absence of auxin and cytokinin, whereas normal cells can be cultured only when these hormones are exogenously supplied.

A. tumefaciens induces phenotypically diverse tumors, depending on both the host plants and the bacterial strains (Gresshoff et al., 1979). For example, the nopaline-type Ti plasmid pTiC58 induces unorganized, tumorous calli on tobacco (*Nicotiana tabacum*) plants, whereas the nopaline-type Ti plasmid pTiT37 produces shoot-forming teratomas on tobacco (Willmitzer et al., 1983). Skoog and Miller (1957) demonstrated that tobacco plant morphology is determined by the ratio of cytokinin to auxin in culture medium; a high ratio is responsible for shoot formation, whereas a low ratio causes root formation, and intermediate ratios lead to unorganized callus formation.

Therefore, the difference in phenotypes induced by *A. tumefaciens* may be ascribed to modulation of the cytokinin/auxin balance by the phytohormone synthesis genes.

Alternatively, distinct tumor morphology could result from the action of the T-DNA-encoded genes other than those for phytohormone synthesis. For example, the gene *5*, which codes for the enzyme required for conversion of Trp to indole-3-lactate, can act as an auxin antagonist (Körber et al., 1991). As another example, the *6b* gene, which is still of unknown function, was shown to induce tumors on intact stems in a limited number of plant species (Hooykaas et al., 1988). Because the *6b* gene per se did not promote growth of *N. tabacum* tissues on tissue culture medium (Spanier et al., 1989), many of the later studies were focused on the effects of the *6b* gene on the activity of the cytokinin and/or auxin genes. Spanier et al. (1989) suggested that the *6b* gene from the octopine-type Ti plasmid pTiAch5 reduced cytokinin gene activity. In contrast, the *6b* gene of the vitopine-type plasmid pTiS4 enhanced both auxin and cytokinin effects (Canaday et al., 1992). Another recent study showed that the pTiTm4 (octopine/cucumopine type) *6b* gene changed the sensitivity of *Nicotiana rustica* protoplasts to phytohormones (Tinland et al., 1992).

Among the diverse nopaline-type Ti plasmids that we isolated previously AKE10 was found to induce only small crown galls on certain plants (Wabiko et al., 1989). Further-

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Abbreviations: AK-*6b*, the *6b* gene of *Agrobacterium tumefaciens* strain AKE10; MS, Murashige-Skoog; *nptII*, encoding neomycin phosphotransferase II; RT, reverse transcriptase; T-DNA, transferred DNA; Ti, tumor-inducing; t-Z, *trans*-zeatin; t-ZR, *trans*-zeatin riboside.

more, none of the auxin and cytokinin genes of pTiAKE10 was identified in the gall tissues of petunia plants induced by the strain, although they were present in poplar crown gall (Wabiko et al., 1991). Such host specificity may reside at least in part in the unique characteristics of the modulating gene(s) of this particular strain. Here we report that a T-DNA segment located outside the auxin and cytokinin genes of the Ti plasmid of AKE10, pTiAKE10, induced shooty callus formation and subsequent maturation of tobacco plants in the absence of exogenous phytohormones in culture medium. The AK-*6b* located in this segment appears to be responsible for this control of plant growth and development.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

Escherichia coli strains JM83 (BRL) and XL1-Blue (Stratagene) were used for preparation of recombinant DNA. *Agrobacterium tumefaciens* strain AKE10 had been isolated previously from crown gall developed on apple trunk (Wabiko et al., 1989). The commonly used disarmed *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) was used for transformation of tobacco plants, *Nicotiana tabacum* cv Xanthi NC (Japan Tobacco, Toyoda). Plasmid vectors pUC19 (Yanisch-Perron et al., 1985) and pBluescriptII SK$^+$ (Stratagene) were used for subcloning the T-DNA in *E. coli*. Plasmid pAKK1 is composed of pUC19 and a 23-kb *Kpn*I fragment corresponding to the T-DNA of pTiAKE10 (Wabiko et al., 1991). The binary plasmid vectors were pGA580 (Pharmacia) and pBI101 (Clontech, Palo Alto, CA), both of which contain the *npt*II gene, which confers kanamycin (300 μg mL^{-1}) resistance on tobacco. *E. coli* was grown on Luria-Bertani medium (Sambrook et al., 1989) and *A. tumefaciens* was grown on either Luria-Bertani or YEB medium (Vervliet et al., 1975). The medium for plant cell culture was composed of MS salts (Wako Pure Chemical, Osaka, Japan), B_5 vitamins (100 mg of *myo*-inositol, 10 mg of thiamine-HCl, 1 mg of nicotinic acid, and 1 mg of pyridoxine-HCl in 1 L), 3% (w/v) Suc, and 0.8% (w/v) agar (BA30, Ina Food, Ina, Japan).

Bacterial Transformation

E. coli was transformed with plasmids in a medium containing PEG as described by Chung and Miller (1988). Transformation of *A. tumefaciens* was performed by electroporation according to the method of Wen-jun and Forde (1989).

Construction of Recombinant Clones

The essential part of the pTiAKE10 T-DNA is located in the 13-kb *Sma*I fragment of pAKK1. This fragment was first subcloned into the *Sma*I site of pUC19 to obtain pAS10 (Fig. 1A). The 3.5-kb *Eco*RI/*Hin*dIII fragment in pAS10 contains the intact AK-*6b* and *3′* genes and is located outside the auxin and cytokinin synthesis genes (Fig. 1A). This fragment was subcloned into *Eco*RI/*Hin*dIII sites of pUC19 to generate pAKEH1. To inactivate AK-*6b* a frame-shift mutation was introduced at the *Bam*HI site, which is located in the middle of the gene (Fig. 1A). Since two *Bam*HI sites exist in pAKEH1 the plasmid was linearized by partial digestion with *Bam*HI, then filled in by the Klenow fragment, and circularized by self-ligation, creating pAKEH7.

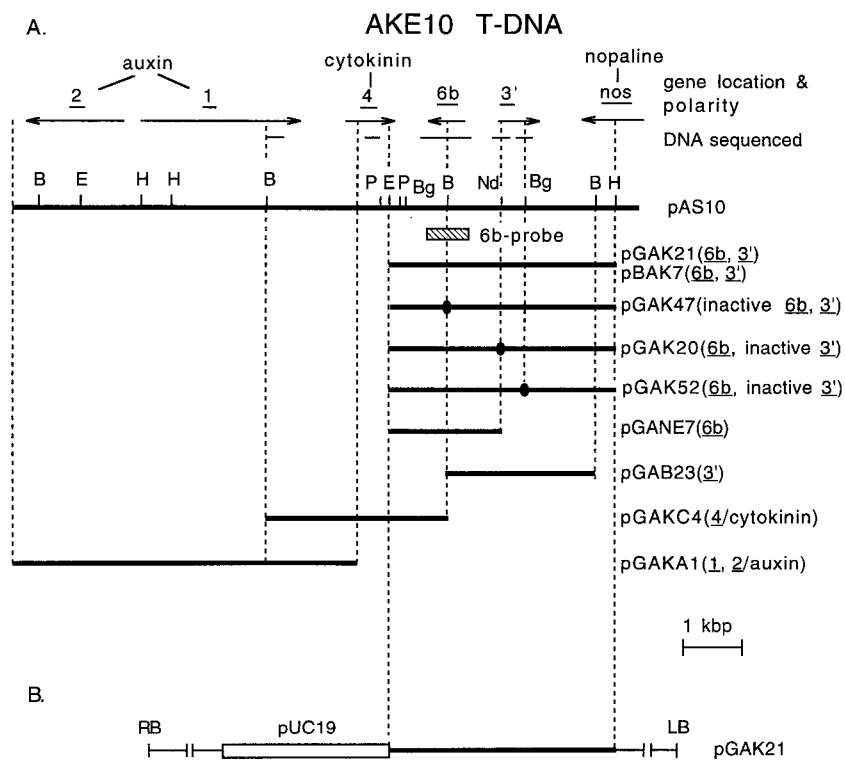

Figure 1. Restriction map of the AKE10 T-DNA segment and the recombinant vectors. A, Restriction map with respect to the AKE10 T-DNA segment of pAS10, which is shown by a wide line. Horizontal thin lines above the map represent regions where the DNA sequence was determined. Size and polarity of transcripts of the indicated genes, shown by arrows, are as determined by Willmitzer et al. (1983) and Drevet et al. (1994) in tumor lines induced by the nopaline-type strains T37 and 82.139, respectively. These genes were aligned to those of the AKE10 T-DNA from a comparative analysis of the restriction enzyme cleavage patterns and nucleotide sequence. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Nd, *Nde*I; P, *Pst*I. The AKE10 T-DNA portions in the individual recombinant clones are also shown by wide lines. Ovoid dots on the T-DNA region depict the filled-in restriction sites. Dashed vertical lines join the corresponding sites shown in this map. The cross-hatched bar indicates the AK-*6b*-specific probe. B, Right (RB) and left (LB) borders flanking the modified T-DNA region in the clone pGAK21. This segment is considered to be transferred into plants.

Similarly, the 3′ gene was inactivated at the *Bgl*II site or *Nde*I site in the coding region, creating pAKEH20 and pAKEH52, respectively. To clone AK-*6b* alone the 1.7-kb *Nde*I/*Eco*RI fragment from pAKEH1 was excised and ligated to the *Nde*I/*Eco*RI sites of pUC19, generating pANE7. To obtain the 3′ gene alone the 2.3-kb *Bam*HI from pAKEH1 was cloned at the *Bam*HI site of pUC19, creating pAB23. These plasmids were linearized by *Hin*dIII digestion and cloned into the unique *Hin*dIII site of the binary vector pGA580, generating pGAK21 (from pAKEH1), pGAK47 (from pAKEH7), pGAK20 (from pAKEH20), pGAK52 (from pAKEH52), pGANE7 (from pANE7), and pGAB23 (from pAB23) (Fig. 1A). The 3.5-kb *Eco*RI/*Hin*dIII insert of pAKEH1 was also cloned into the *Eco*RI and *Hin*dIII sites of pBI101, and pBAK7 was obtained (Fig. 1A).

The intact cytokinin biosynthesis gene (gene 4) was included in the 2.7-kb *Bam*HI fragment (Fig. 1A). This segment was cloned into the *Bam*HI site of pUC19 to generate pAS10B7. The 2.7-kb *Sma*I (in pUC19)/*Hin*dIII (in pUC19) fragment of pAS10B7 containing gene 4 was ligated to pGA580, which had been digested with *Kpn*I, made blunt-end by T4 DNA polymerase, and then cut with *Hin*dIII, creating the clone pGAKC4 (Fig. 1A). Gene 4 was also cloned from pTiPO22 of the nopaline strain PO22 (Wabiko et al., 1989) as follows. The intact gene was included in the 1.9-kb *Bam*HI/*Pst*I fragment of the T-DNA clone PO22P1 (Wabiko et al., 1991). This fragment was first cloned into pUC19 and then excised by double digestion with *Hin*dIII and *Sma*I (both cleavage sites flanking the insert are located in pUC19). The resulting 1.9-kb fragment was ligated to *Hin*dIII/filled-in-*Kpn*I (blunt end) sites of the vector pGA580, generating the plasmid pGPOC4. To obtain the auxin biosynthesis genes, pAS10 was first digested with *Pst*I. The major part of the coding region of gene 4 was then deleted by treatment with nuclease Ba131 from the *Pst*I site, which is located 389 bp downstream of the initiation codon of gene 4 (Fig. 1A). The deleted DNA was filled in by the Klenow fragment, digested with *Kpn*I (located in pUC19), and then ligated to pUC19, which had been digested with *Sma*I and *Kpn*I, thus generating pAKS88. The deletion end point of pAKS88 corresponds to 170 bp downstream of the initiation codon of gene 4 as determined by sequencing. The 12-kb *Kpn*I/filled-in-*Hin*dIII (blunt) fragment of pGA580 was ligated to the 6.5-kb *Kpn*I/filled-in-*Pst*I (blunt) fragment of pAKS88 containing the intact *1* and *2* genes, thus creating pGAKA1 (Fig. 1A). These recombinant plasmids, composed of various T-DNA segments and vectors pGA580 or pBI101, were introduced into LBA4404 and used for plant transformation.

Transformation and Regeneration of Tobacco

Young leaves of tobacco plants were surface-sterilized in 1.2% (v/v) sodium hypochlorite for 5 min. Leaf discs (0.5 × 0.5 cm) were infected with *A. tumefaciens* LBA4404 carrying the recombinant vectors at a density of 10^7 cells mL^{-1} for 1 min. The discs were blotted onto filter paper to remove excess bacterial cells and then placed onto MS medium for 48 h under constant light at 27°C. The infected leaf discs were then transferred to MS medium containing additional kanamycin (300 $\mu g\ mL^{-1}$), carbenicillin (500 $\mu g\ mL^{-1}$), and cefotaxime (500 $\mu g\ mL^{-1}$) without auxin or cytokinin. After several passages through that medium, tissues were transferred to the same medium without carbenicillin and cefotaxime to ensure the absence of bacteria. Spontaneously developed stems with normal leaves were periodically transferred to MS medium or to one-half-concentration MS medium with kanamycin (300 or 100 $\mu g\ mL^{-1}$) to allow root formation. Rooted plantlets were transferred to soil and cultivated under a 16-h light/8-h dark photoperiod at 27°C and 70% humidity.

The shoot-forming callus PC1 was established by the introduction of the cytokinin synthesis gene 4 upon infection with LBA4404 harboring pGPOC4 and subsequent culturing on hormone-free MS medium. A control transgenic plant, expressing only the *npt*II gene, was obtained by transforming leaf discs with LBA4404 carrying the vector pGA580 and selection on MS medium containing BA (1.0 $\mu g\ mL^{-1}$), NAA (0.1 $\mu g\ mL^{-1}$), and kanamycin (300 $\mu g\ mL^{-1}$) as the standard selective medium (Rogers et al., 1986).

DNA Isolation and Southern Hybridization

Total plant DNA was prepared by the cetyltrimethylammonium bromide method of Murray and Thompson (1980). DNA was digested with the appropriate restriction enzymes, fractionated by 0.8% (w/v) agarose gel electrophoresis, and then blotted onto a nylon membrane (GeneScreen*Plus*, DuPont). Southern hybridization was performed at 42°C in the presence of 50% formamide as described previously (Wabiko et al., 1990) using a ^{32}P-radioactive probe labeled by the random-primer method (DuPont). Hybridization was followed by high-stringency washing in 0.1× SSC, 0.1% SDS at 65°C. The AK-*6b*-specific probe spans from 2 bp upstream of the initiation codon through 146 bp downstream of the termination codon. The probe DNA was amplified by PCR using one of the deletion clones created for DNA sequence analysis as a template, the AK-*6b*-specific primer (19-mer 5′-AAATGACGGTTCCTACTTG-3′), and the pBluescriptII-specific KS primer, which is situated adjacent to the deletion end point.

RNA Isolation and Northern Hybridization

Total RNA from plant tissues was extracted by the SDS/phenol method, precipitated by 2 M LiCl, and collected by centrifugation through 5.7 M CsCl (Ausubel et al., 1987). Poly(A)$^+$ RNA was further purified by oligo(dT)-cellulose column chromatography (Sambrook et al., 1989). Total RNA was denatured, separated on a formaldehyde-1% agarose gel, and blotted onto a nylon membrane (Hybond N, Amersham), as described previously (Sambrook et al., 1989). Subsequent hybridization using the AK-*6b*-specific probe was performed as in Southern hybridization analysis, followed by high-stringency washing conditions.

RT-PCR Analysis

To identify low levels of AK-*6b* transcripts, the cDNA was amplified by PCR according to the procedure of Frohman et al. (1988). A 1-μg aliquot of poly(A)$^+$ RNA

was used as the template and treated with RAV-2 reverse transcriptase (Takara, Tokyo, Japan) in a 20-μL reaction mixture, as described previously (Sano and Youssefian, 1991). A 29-base adaptor-primer was used with sequence 5′-CAATTCGCGGCCGC$(T)_{15}$-3′, in which GCGGCCGC is a *Not*I cleavage site. After first-strand cDNA synthesis the mixture was brought to 100 μL by the addition of H_2O. A 5-μL sample was amplified by PCR in a 50-μL mixture containing 1× *Taq* polymerase buffer, 2.5 mM $MgCl_2$, 200 μg mL^{-1} gelatin, 0.2 mM deoxyribonucleotide triphosphate, 23 pmol of the adaptor primer (29-mer), 30 pmol of the above AK-*6b*-specific primer (19-mer), and 2.5 units of *Taq* polymerase (Kurashiki-Bōseki, Tokyo, Japan). Denaturation, annealing, and extension reactions were: 94°C for 3 min (1 cycle), then 94°C for 1 min, 50°C for 1 min, 72°C for 1 min (30 cycles), and 72°C for 7 min (1 cycle).

DNA Sequence Analysis

Deletion clones suitable for DNA sequence analysis were generated from the 3.5-kb *Eco*RI/*Hin*dIII fragment of pAKEH1 using nuclease Ba131 from both the *Eco*RI and *Hin*dIII sites and cloned into the vector pBluescriptII SK^+. The DNA sequence was determined by the dideoxy-chain termination method of Sanger et al. (1977) using Sequenase version 2.0 (United States Biochemical) and analyzed by GENETYX software (Software Development, Tokyo, Japan).

Cytokinin Determination

A 5-g tissue sample was ground in liquid nitrogen with a mortar and pestle, and cytokinins were extracted in 50 mL of 80% methanol on ice for 2 h. Extracts were clarified by centrifugation (10,000*g* for 10 min), and the precipitate was washed once with 10 mL of 80% methanol. The soluble supernatants were combined, and the volume was reduced to about 20 mL using a rotary evaporator. To remove phenolic compounds, the extracts were added to a suspension of polyvinylpolypyrrolidone (0.5 g, Sigma) equilibrated in 2 mL of water (adjusted to pH 3.5 with dilute acetic acid). The mixture was shaken for 20 min and clarified by centrifugation (8,000*g* for 15 min), and the process was repeated by treatment of the supernatant with a new batch of polyvinylpolypyrrolidone. The supernatants were combined, evaporated to dryness, and resuspended in 10 mL of 10 mM triethylammonium acetate at pH 7.0 (adjusted by dilute acetic acid). The sample was passed through a C_{18} cartridge (Sep-Pak Plus, Millipore) according to the method of Smart et al. (1991), eluted with 10 mL of 50% methanol, and dried completely. It was then dissolved in 110 μL of 15 mM potassium phosphate buffer (pH 6.5) and used for the cytokinin-induced betacyanin bioassay in *Amaranthus caudatus* L. according to the method of Biddington and Thomas (1973). Alternatively, after passage through the cartridge the dried material was dissolved in 50 μL of 20% (v/v) methanol and separated by HPLC, using a C_{18} column (Puresil, Waters) under isocratic conditions with 20% methanol. Individual 1-mL fractions were dried completely and assayed for t-Z and t-ZR by ELISA. With this method the t-Z and t-ZR in samples were allowed to bind to a monoclonal antibody raised against t-ZR (Idetek, San Bruno, CA) competitively, with t-ZR conjugated with alkaline phosphatase. Conjugation had been accomplished by the periodate oxidization method (Ausubel et al., 1987). Fluorogenic 4-methylumbelliferyl phosphate at 1 mg mL^{-1} was used as a substrate of alkaline phosphatase (Trione et al., 1987).

Tumorigenicity Assay

Stems of intact plants in soil were punctured by toothpicks that had been immersed in *A. tumefaciens* grown to about 10^8 cells mL^{-1} in Luria-Bertani medium containing the appropriate antibiotics. Infected plants were allowed to grow under the conditions described, and tumor development was monitored after 3 months.

RESULTS

Structure of AKE10 T-DNA

Partial DNA sequence analysis of the intragenic region of gene *1* (auxin) and gene *4* (cytokinin) and the flanking region of gene *2* (auxin) of pTiAKE10 T-DNA revealed a high degree of similarity (84%) with the homologous genes from pTi15955 and pTiAch5 (Fig. 1A). This result confirms the previous finding that the structure and organization of the auxin and cytokinin biosynthesis genes of pTiAKE10 are closely related to those of pTi15955 and pTiT37, as determined by Southern hybridization analysis (Wabiko et al., 1991).

To determine the structure of the region containing the *6b* gene, pAKEH1 (from pTiAKE10) was digested with restriction enzymes *Bam*HI, *Bgl*II, *Nde*I, and *Nru*I, and the restriction pattern was compared with that from known T-DNA. The released fragments possessed the same sizes as those expected from pTi82.139 of the nopaline-type strain 82.139 (Drevet et al., 1994). Furthermore, the DNA sequence of the coding and the flanking regions of the pTiAKE10 *6b* gene were identical with the corresponding segments of pTi82.139 and very similar (97%) to those of pTiAB4 from the nopaline strain *A. vitis*. The *3′* gene sequence from pTi82.139 showed 90% identity with that from pTiAKE10. From these analyses, we concluded that the 3.5-kb *Eco*RI/*Hin*dIII fragment contained the complete *6b* and *3′* genes and most likely part of gene *4* and the *nos* gene (Fig. 1A). The structure of this region was nearly identical with that of pTi82.139.

Tobacco Regeneration on Hormone-Free Medium

Leaf discs of normal tobacco plants were infected with *A. tumefaciens* LBA4404, carrying various recombinant plasmids containing the 3.5-kb *Eco*RI/*Hin*dIII T-DNA fragment or its derivative segments located outside the auxin and cytokinin biosynthesis genes of strain AKE10 (Fig. 1A). The infected discs were cultivated on hormone-free MS medium containing kanamycin. Incorporation of kanamycin into the medium allowed selection for transformants ex-

pressing the *npt*II gene from the vector, and elimination of auxin and cytokinin from the medium allowed us to examine the effects of the T-DNA segments on plant growth. As summarized in Table I, approximately 3 weeks after infection with bacteria harboring pGAK21 or pBAK7 and containing intact *6b* and *3'* genes, tiny calli appeared from 6 to 20% of the total leaf discs. As the calli developed they turned greenish (Fig. 2A) and formed protruding structures, which appeared to be incomplete leaves, at numerous portions of the calli (Fig. 2B, arrows). Control leaf discs, which were either uninfected or infected with the strain harboring the vector pGA580, did not form any calli. To determine whether the *6b* and/or *3'* genes were involved in this callus formation frame-shift mutations were generated in the coding regions of the *6b* gene (in pGAK47) and the *3'* gene (in pGAK20 and pGAK52) and were used to transform tobacco under the same conditions. Calli did not develop into stable green calli using pGAK47 carrying inactive *6b* but active *3'* genes, although a few small calli did appear in only one experiment (Table I). In contrast, shooty calli appeared from discs transformed with pGAK20 and pGAK52, which contained active *6b* but inactive *3'* genes. Furthermore, the clone pGANE7, containing the *6b* gene alone, produced shooty calli, whereas clone pGAB23 containing the *3'* gene alone did not (Table I). These results indicate that AK-*6b* is required and sufficient for stable callus formation in the absence of exogenous phytohormones.

Southern Hybridization Analysis

The presence of the T-DNA segments in the pGAK21-induced tumorous calli was examined by Southern hybridization analysis. Total DNA was prepared from shoot-forming tissues (K4, K5, K6, K16, K17, and K20), which had been cultured for about 3 months on hormone-free MS medium with kanamycin under axenic conditions. The DNA was double-digested with *Eco*RI and *Hin*dIII and then hybridized to the AK-*6b*-specific probe. Hybridization of a single 3.5-kb fragment was identified in all DNA samples (Fig. 3, top) except for the K4 plant, which contained an additional 6.4-kb fragment that may have resulted from rearrangement during integration into the plant genome. The 3.5-kb fragment size agreed well with the *Eco*RI/*Hin*dIII fragment obtained from vector pGAK21, demonstrating that the T-DNA segment had been transferred intact into the plant chromosome. To examine the approximate number of T-DNA integration sites total DNA was digested with *Bam*HI and probed with pUC19, which is part of the transferred DNA from pGAK21. Of the two *Bam*HI sites flanking the pUC19-containing fragment, one was located in the 3.5-kb T-DNA segment and the other was located outside the right border of T-DNA, being derived from plant DNA (Fig. 1B). Thus, the number of hybridized fragments gives an estimate of the number of T-DNA segments in plants, assuming that each copy integrates into the plant chromosome at a unique site. Single to multiple signals were identified, depending on the particular plant clone (Fig. 3, bottom), and this was consistent with an earlier report demonstrating that full-length T-DNA in plants exists from single to multiple loci (Thomashow et al., 1980).

Regeneration Potential of Transgenic Tobacco

To identify possible effects of AK-*6b* on plant growth and development we examined the regeneration potential of transformed tobacco tissues. The massive shoot-forming calli were maintained with numerous passages through hormone-free MS medium. Of eight shooty calli initially induced by pGAK21 five plants (including K4, K5, K6, and K20) developed into elongated shoots concurrently with maturation of

Table I. *Callus formation and regeneration of tobacco plants induced by AK-6b*

Tobacco leaf discs were infected with LBA4404 carrying individual clones and callus formation was monitored on hormone-free MS medium with kanamycin (300 μg mL^{-1}), carbenicillin (500 μg mL^{-1}), and cefotaxime (500 μg mL^{-1}).

Clones and Genotypes[a]	No. of Discs Tested[b]	No. of Callus-Forming Discs			Mature Plants
		Small (3 weeks)[c]	Large/green (1 month)	Shooty (3 months)	
LBA4404					
pGAK21/*6b,3'*	45	8	8	8	5
	45	9	8	8	ND[d]
	17	2	2	2	ND
pBAK7/*6b,3'*	77	5	5	5	ND
pGAK47/*6b*$^-$,*3'*	22	2	0	0	0
	27	0	0	0	0
pGAK20/*6b,3'*$^-$	42	4	3	3	1
pGAK52/*6b,3'*$^-$	33	2	1	1	ND
pGANE7/*6b*	60	13	4	4	4
pGAB23/*3'*	52	0	0	0	0
pGA580/vector	15	0	0	0	0
	36	0	0	0	0
	50	0	0	0	0
No infection	24	0	0	0	0
	43	0	0	0	0

[a] *6b*$^-$ and *3'*$^-$ indicate that these genes were inactivated. [b] Results from each row represent separate experiments. [c] Elapsed time after infection is shown in parentheses. [d] ND, Not determined.

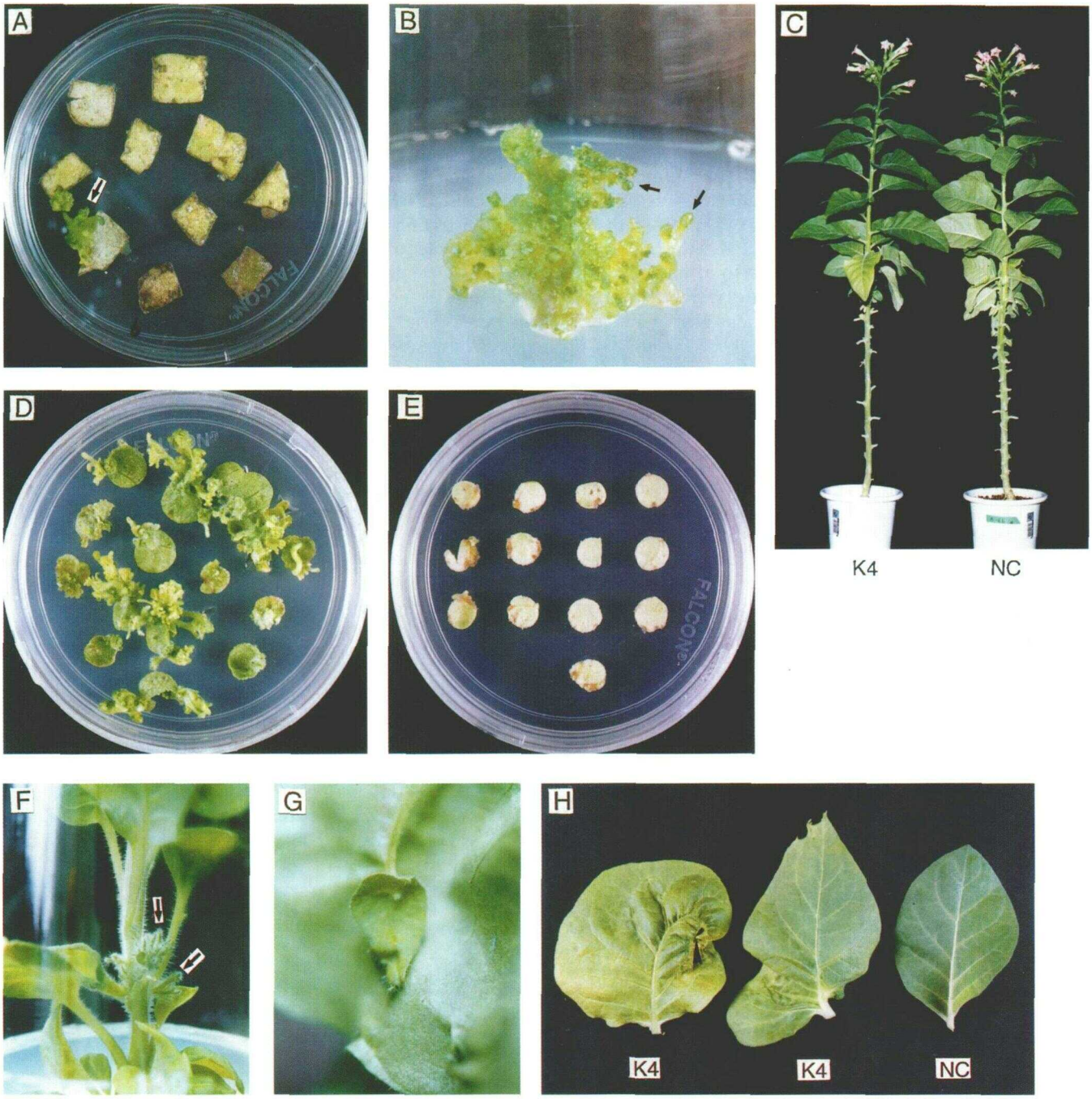

Figure 2. Transformation and regeneration of tobacco plants. A, Callus formation by pGAK21. Leaf discs were infected with LBA4404 harboring pGAK21 containing the wild-type AK-*6b* and selected on MS medium containing carbenicillin (500 μg mL^{-1}), cefotaxime (500 μg mL^{-1}), and kanamycin (300 μg mL^{-1}). The photograph was taken 4 weeks after infection. The arrow indicates shoot-forming calli. B, Shooty calli excised from transformed leaf discs. The calli were allowed to grow on MS medium with kanamycin (300 μg mL^{-1}) for 3 months after infection. The arrows indicate protruding immature leaves. C, Mature transgenic and control plants. Transgenic plant (K4), obtained by infection with LBA4404 (pGAK21), and a normal control, Xanthi NC (NC), were grown to maturity. D, Increased regeneration potential of the transgenic K4 plant. Leaf discs of the K4 plant were placed onto MS medium containing kanamycin (300 μg mL^{-1}) without phytohormones and then photographed 1 month later. E, The same experiment as in D was performed using a control GA1 plant containing the *npt*II gene alone from the vector pGA580. F, Abnormal shoot formation on MS medium. When the transgenic K4 plant reached 5 to 6 cm in height with mature leaves and roots on MS medium, the plant was decapitated and then cultured for an additional 1 month on the same medium. Arrows show the abnormally thin shoots. G, Abnormal leaf development. Development of a new leaf on the vein of the abaxial surface of a leaf of the K4 plant grown in soil. H, Abnormal leaf morphology. Abnormal leaves, asymmetric along the central vein, developed on the K4 plant grown in soil. An arrowhead points to the leaf end. NC, Normal leaf of Xanthi NC grown in soil.

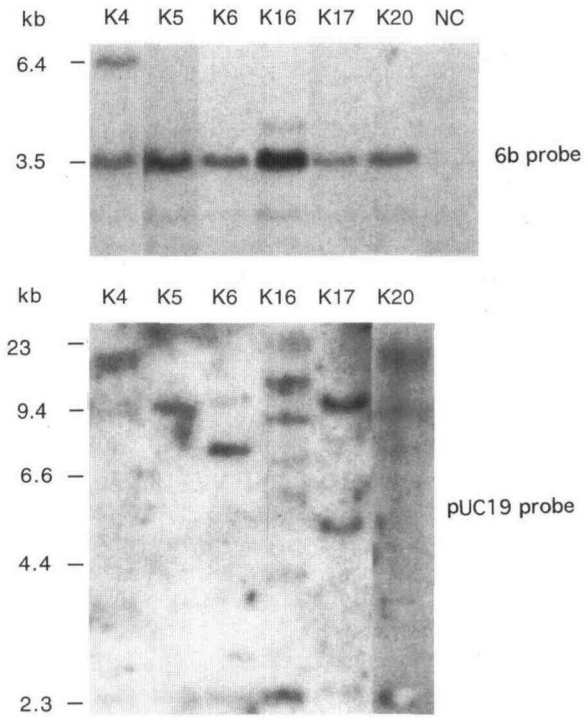

Figure 3. Southern hybridization analysis of the transformed tobacco DNA. Top, Total DNA (10 μg) from individual tissues was double-digested with *Eco*RI and *Hin*dIII and separated by 0.8% agarose gel electrophoresis. The fragments were transferred to a nylon membrane and hybridized to a ^{32}P-labeled AK-*6b*-specific probe (3×10^7 cpm, 6×10^8 cpm μg^{-1} DNA). K4 to K19 indicate the individual transformed plants, and NC indicates the control Xanthi NC plant. Bottom, Similar hybridization was performed except that the plant DNA was first digested with *Bam*HI and then probed with labeled pUC19. Molecular size markers shown on the left were obtained by λDNA/*Hin*dIII digests.

leaves, whereas three plants (including K16 and K17) remained as shooty calli. Likewise, one of three shooty calli induced by pGAK20 and all four (including an NE1 plant) induced by pGANE7 developed to mature plants.

Efficient root formation was accomplished by successively transferring shoots to MS or to one-half-strength MS medium. At this stage the shape and stature of the transgenic tobacco appeared to be normal compared with the wild-type plant.

To further examine the regeneration potential of the transgenic tobacco, four independently isolated transgenic plants (K4, K5, K6, and K20) generated by pGAK21 and one plant (NE1) generated by pGANE7 were grown on hormone-free MS medium. Leaf discs were excised from these plants and placed onto the same medium. After 2 weeks calli with shoots appeared at the wounding sites from all plants and 1 month later the tiny shoots developed into massive shoots that were composed of many thin branches (Fig. 2D), but leaf development was not obvious in many shoots. When the upper portion of the stem was removed by cutting, lateral buds immediately below the decapitated site grew normally, whereas abnormally thin shoots with tillers developed from the wounded sites (Fig. 2F). Similarly, greenish shooty calli developed from the wounded sites of excised roots (data not shown). Some of the immature shoots again became normal, mature shoots by subculturing, and excised tissues showed the same response on hormone-free medium as above. In contrast, neither callus nor shoots developed from leaf discs of the control transgenic plant GA1 obtained by transformation with only vector pGA580 (Fig. 2E).

When the mature K4 plant was transferred to soil, growth and flowering were indistinguishable from normal plants (Fig. 2C), and seeds set normally. However, a close examination revealed that several leaves of the mature plants showed morphological abnormalities. For example, small but distinct leaves developed from the veins of the abaxial surface of the main leaves (Fig. 2G). Some leaves were extremely asymmetric along the central vein (Fig. 2H). Excessive leaf formation was also observed along the veins in the K5 plant (data not shown). The M_1 seeds obtained from the K4 plant germinated normally on hormone-free MS medium containing kanamycin, and the seedlings showed no abnormalities. However, leaf discs excised from 13 independently selected seedlings produced massive shoots on hormone-free MS medium as in the parental K4 plant, indicating that the shoot-forming phenotype was heritable. One of these M_1 plants, which was arbitrarily selected, was found to contain a 3.5-kb T-DNA segment but lacked the rearranged 6.4-kb fragment present in the K4 plant as a result of segregation. This indicated that regeneration capacity is not due to DNA rearrangement. From these results we concluded that the transgenic plants containing AK-*6b* possessed highly regenerative potential compared with normal plants.

Expression of AK-*6b*

To investigate AK-*6b* expression during shoot development the AK-*6b* transcript levels in transformed tobacco at different stages of development on MS medium were examined by northern hybridization analysis using the AK-*6b*-specific probe. A single discrete hybridization signal of approximately 0.9 kb was observed in shoot-forming calli (Fig. 4). Transcript size was consistent with the 626-bp coding region of AK-*6b* deduced from its DNA sequence. Since the 3.5-kb T-DNA *Eco*RI/*Hin*dIII segment contains another gene (*3'*) that is situated upstream of AK-*6b*, and is transcribed in an opposite direction to AK-*6b* (Drevet et al., 1994; Fig. 1A), AK-*6b* was most likely transcribed from its own promoter. In contrast, no transcripts were observed in mature shoots (stem and leaves) that had developed normally from the shooty calli (Fig. 4) or that had been grown in soil (data not shown). A limited amount of transcript was observed in roots of the normally developed K4 plant on MS medium (Fig. 4). Similar experiments performed with leaf discs placed on MS medium for up to 37 h showed no accumulation of AK-*6b* transcripts (data not shown). Nor was hybridization detected after leaf discs were treated with auxin or cytokinin (data not shown). These results suggest that simple wounding, nutritional supplementation of MS medium, auxin, and cytokinin are not responsible for the induction of AK-*6b* transcript accumulation.

To examine low levels of AK-*6b* transcript in the mature plant in soil we used RT-PCR analysis. Poly(A)$^+$ RNA was annealed to a poly(dT)-adaptor and cDNA was synthesized

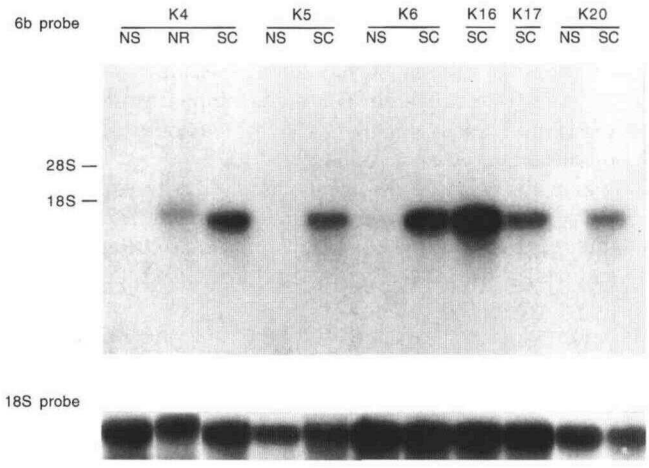

Figure 4. Northern hybridization analysis of transformed tobacco. Top, Total RNA (50 μg) was separated by formaldehyde/1% agarose gel electrophoresis and analyzed by northern hybridization using an AK-*6b*-specific probe as in Southern hybridization. NS and NR indicate RNA samples that were obtained from shoots and roots, respectively, of the phenotypically normal, mature plants grown on MS medium with kanamycin, and SC denotes RNA obtained from shoot-forming callus tissues cultured on the same medium. Bottom, The same filter was rehybridized with a cDNA probe encoding the 18S rRNA of *A. thaliana* to standardize loading.

with RT. The K4 plant cDNA was PCR-amplified using the AK-*6b*-specific primer and the adaptor primer (see "Materials and Methods") and analyzed by agarose gel electrophoresis. Ethidium bromide staining showed no distinct amplified DNA band, and DNA in the gel was consequently transferred onto a nylon membrane and subjected to Southern hybridization analysis using the AK-*6b*-specific probe and using a relatively prolonged exposure of the hybridized filter to x-ray film. Three weak hybridization signals were identified in the 0.8-kb region but were absent when the RT was omitted from the cDNA synthesis reaction (Fig. 5). These results indicate that the accumulation of AK-*6b* transcripts is high in shoot-forming callus tissues cultivated on MS medium but extremely low in mature plants.

Intracellular Cytokinin Levels in Transgenic Tobacco

Since it is well established that excess cytokinin promotes shoot organogenesis, extensive shoot regeneration from leaf discs of the transgenic tobacco might have resulted as a consequence of the increased intracellular concentration of cytokinin. To test this possibility active cytokinin levels of the transgenic plants were determined by a bioassay in which cytokinin-induced betacyanin production was measured in *Amaranthus* seedlings. The cytokinin gene-induced shoot-forming callus PC1 contained the highest cytokinin level (Table II), whereas the shoot-forming calli of the AK-*6b* transgenics K4 and K5 had only limited amounts (Table II). Cytokinin levels of the K4 and K5 shooty calli were comparable to those of the mature, normal shoots of the K4 plant and the wild-type tobacco plant and were close to the limit of detection (0.8–2.4 pmol g^{-1} tissue) under the current bioassay system. To exclude the possibility that the apparently low levels of active cytokinin could be due to the presence of inhibitory substances in the extracts or to enzymatic activities that degrade cytokinins, known amounts of t-Z were mixed with extracts and the recovery was determined by the bioassay. When t-Z was added in the bioassay along with extracts of wild type, K4/shooty calli, or K5/shooty calli no inhibitory effects were observed (Table II). When t-Z was added immediately after the tissues were extracted with 80% methanol recoveries ranged from 60 to 81% in the final extracts (Table II). These values are within the normal range of t-Z recovery.

To determine t-Z and t-ZR levels in the tissues more precisely extracts were separated by HPLC and individual fractions were immunologically assayed by ELISA using a monoclonal antibody to t-ZR. The cytokinin-overproducing PC1 tissues contained high levels of both t-Z and t-ZR (32 and 21 pmol g^{-1} tissue, respectively), whereas all of the wild-type, K4/normal, K4/shooty, and K5/shooty tissues contained comparable, low levels of these cytokinins (0.86–1.5 pmol g^{-1} tissue for t-Z and 0.27–0.56 pmol g^{-1} tissue for t-ZR, Table II). Therefore, the bioassay and physical determination of cytokinins consistently supported the conclusion that the shooty calli of the AK-*6b* transgenic plants contained only normal levels of active cytokinins.

Effects of Auxin and Cytokinin Genes on the Transgenic Tobacco

To examine the effect of auxin and cytokinin on AK-*6b* action stems of the transgenic plants grown normally in soil were infected with LBA4404 harboring individual clones, pGAKA1 containing the auxin genes (*1* and *2*), or pGAKC4 containing the cytokinin gene (*4*) (Fig. 1A). Both pGAKA1 and pGAKC4 induced only tiny tumorous growth on wild-type tobacco, and sometimes even this outgrowth was not evident. No response was observed in wild-type plants after

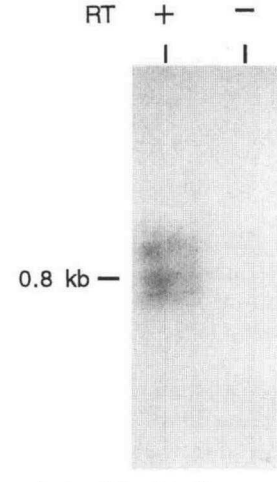

Figure 5. RT-PCR analysis of the AK-*6b* transcript. A poly(A)$^+$ RNA sample obtained from the mature K4 plant grown in soil was used for cDNA synthesis with (+) or without (−) RT and was then subjected to PCR amplification. A 5-μL aliquot of the 50-μL PCR reaction mixture (see "Materials and Methods") was separated by 0.8% agarose gel electrophoresis and analyzed by Southern hybridization using the AK-*6b*-specific probe. Washed membrane filters were subjected to autoradiography for 10 d.

Table II. *Active cytokinin levels of wild-type and AK-6b transgenic tobacco*

Shooty calli (PC1) and leaf discs of K4 and K5 plants were cultured on hormone-free MS medium for 3 weeks and transferred to the same, fresh medium for an additional 1 week. At this stage leaf discs of K4 and K5 developed a shoot-forming phenotype (K4/shooty, K5/shooty). Wild-type shoot (Xanthi NC) and phenotypically normal shoot (K4/normal) developed from shooty calli of the K4 plant were similarly subcultured on the same hormone-free MS medium. Extracts were prepared from these tissues and cytokinins were determined by the bioassay and HPLC/ELISA procedure. Values are expressed in amounts equivalent to t-Z, which was used as the standard. To determine the recovery of t-Z in the presence of extracts known amounts of t-Z were mixed with individual extracts immediately prior to the bioassay (at 360 pmol g^{-1} tissue) or immediately after tissues were extracted with 80% methanol (at 3600 pmol g^{-1} tissue).

Tissue Type	Bioassay					HPLC/ELISA	
	Cytokinin content		Recovery of input t-Z			t-Z	t-ZR
	Experiment 1[a]	Experiment 2[a]	t-Z added in bioassay	t-Z added upon extraction			
				Experiment 1	Experiment 2		
	pmol g^{-1} tissue equivalent to t-Z		%	%		*pmol g^{-1} tissue*	
Wild type	2.8	3.9	96	64	81	0.86	0.50
PC1	154	151	ND[b]	ND	ND	32	21
K4/normal	3.7	ND	ND	ND	ND	1.5	0.56
K4/shooty	1.2	4.2	111	60	ND	0.99	0.27
K5/shooty	3.6	2.8	96	ND	ND	1.2	0.29

[a] Extracts were prepared from two independently cultured tissues (Experiments 1 and 2) of each tissue type. [b] ND, Not determined.

simple wounding. In contrast, both of these clones induced large tumors on both the K4 and K5 plants, except that relatively small tumors were produced by pGAKC4 on the K4 plant (Fig. 6A). The size of large tumors was comparable to that induced on the same plants induced by the wild-type, pathogenic AKE10 strain. Wounding without bacterial infection induced tiny tumors on K5 stems but similar tumorous growth was not apparent in the K4 plant.

To analyze further the properties of the resulting tumorous tissues, they were excised from the plants, surface-sterilized, and placed onto hormone-free MS medium containing kanamycin and cefotaxime. After successive transfer onto the same medium to accomplish an axenic condition, the steady-state AK-*6b* transcript accumulation was found to be similar to that identified in the shoot-forming calli of the parental transgenic K4 and K5 plants (data not shown). At this stage the resulting tissues displayed distinct phenotypes. The auxin gene-induced tumors were composed primarily of unorganized calli with concomitant development of several roots and, therefore, the shoot-forming phenotype directed by AK-*6b* was not apparent (Fig. 6B). Since it is known that auxin is responsible for root formation, AK-*6b* evidently promoted activity of the auxin genes. The cytokinin gene-induced tumors developed to shoot-forming calli (Fig. 6B), which, in contrast to the spontaneous maturation of shoots in the AK-*6b* trangenic plants, did not elongate to make mature stems or produce roots. As such stunted shoots are a typical symptom of the action of the cytokinin gene, this suggests that AK-*6b* also promoted activity of the cytokinin gene.

DISCUSSION

Exogenous Hormone-Independent Plant Growth and Development

In this study we showed that a 3.5-kb T-DNA segment lacking the auxin and cytokinin genes of the strain AKE10 induced callus formation and subsequent shoot regeneration of tobacco plants on hormone-free medium. The DNA segment, which was stably incorporated into the plant genome, induced relatively immature and abnormal shoots. In addition, mature leaves of transgenic plants possessed a high regenerative potential for shoots on the same hormone-free medium. Although the 3.5-kb segment contained both AK-*6b* and 3′ genes, mutagenesis and subcloning experiments indicated that AK-*6b* was sufficient to support plant growth and differentiation. The fact that the 3′ gene was not required for plant regeneration is consistent with the observation that a mutation in the pTi15955 open reading frame 21 gene, which closely resembles the 3′ gene (Drevet et al., 1994), did not affect the oncogenicity of *A. tumefaciens* (Salomon et al., 1984).

Our results are different from previous findings that, in the absence of phytohormones in the tissue culture medium, stable callus formation was not established in *N. tabacum* by the Ach5-*6b* gene (Spanier et al., 1989) or in *N. rustica* or *Daucus carota* by the Tm4-*6b* gene (Tinland et al., 1990). Such conflicting results about the tumorigenic activity and different host-plant specificities of the *6b* genes may reside in their protein sequence divergence. A comparison of the deduced amino acid sequences indicates that the AK-*6b* polypeptide shows a 52 to 55% amino acid identity with those from various different strains, T37, Ach5, Tm4, and S4 (data not shown). Likewise, the S4-*6b* polypeptide shows a 55% identity with those of the other origin. In contrast, amino acid identity is considerably higher (72–90%) among the T37-, Ach5-, and Tm4-*6b* genes. The activity of the S4-*6b* gene is reported to be oncogenic on intact stems of *N. tabacum*. As shown in our findings, this particular plant species appears to be a suitable host for the S4-*6b* gene and AK-*6b*. In contrast, neither the Ach5- nor Tm4-*6b* genes promoted growth of *N. tabacum*. These results suggest some correlation between phylogenic relatedness and tumorigenicity on *N. tabacum*. Clearly, similar experiments are required in different plant systems to confirm our findings.

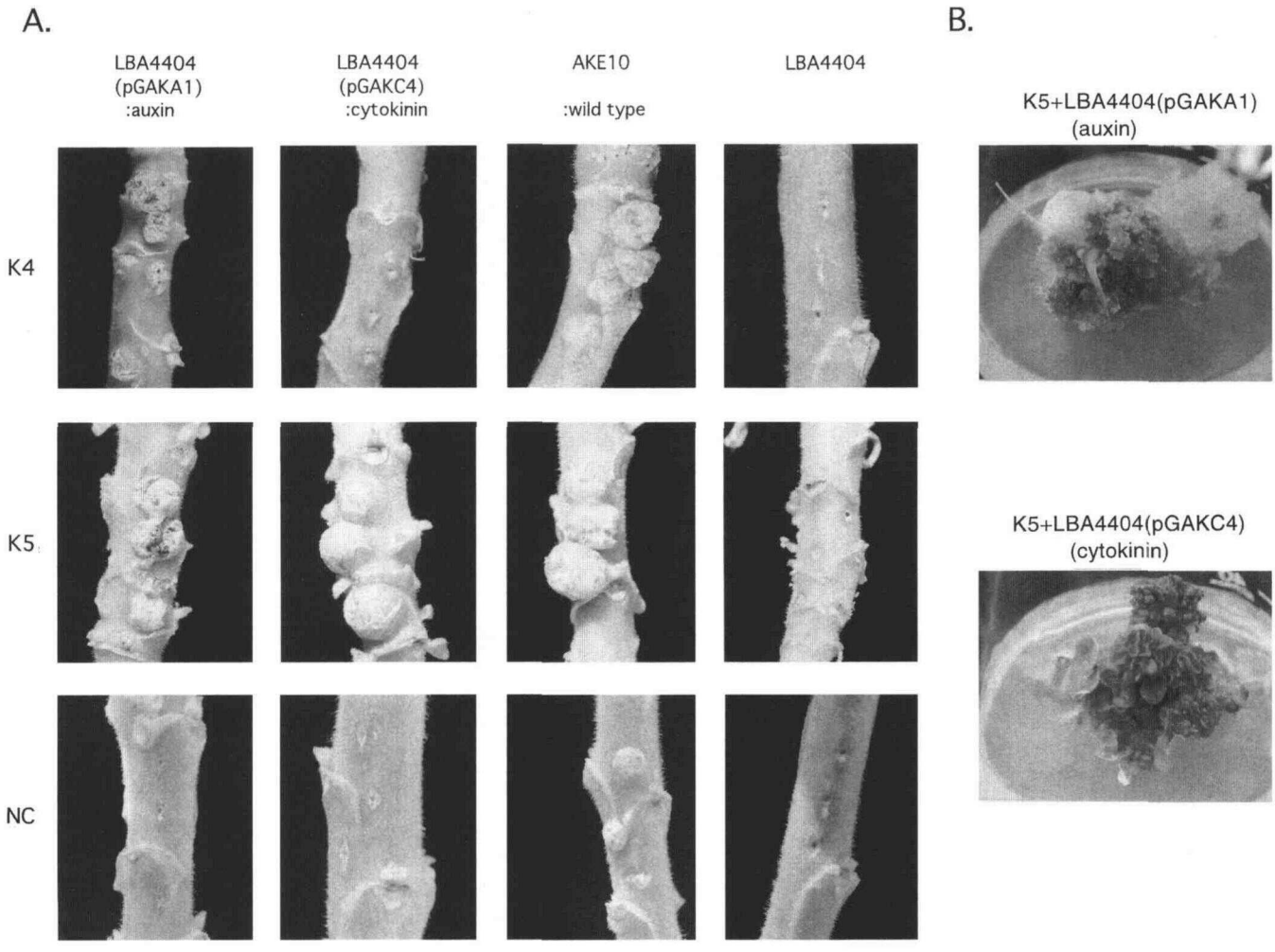

Figure 6. Tumorigenic activity of the transgenic tobacco induced by auxin and cytokinin genes. A, Stems of the young plants K4, K5, and NC (wild-type Xanthi NC) grown in soil were infected with LBA4404 harboring pGAKA1 (containing auxin genes *1* and *2*), or pGAKC4 (containing cytokinin gene *4*), or infected with the wild-type pathogenic strain AKE10. Tumor formation was monitored 3 months after infection. LBA4404 without any recombinants was used as a negative control. B, Tumors, developed on the K5 plant upon infection with LBA4404 (pGAKA1/auxin genes; top) or LBA4404 (pGAKC4/cytokinin gene; bottom), were excised, surface-sterilized, and placed onto hormone-free MS medium containing kanamycin (300 μg mL^{-1}) and cefotaxime (500 μg mL^{-1}). The photograph was taken after successive subculturing for 6 months.

Developmental Regulation of AK-*6b* Expression

We observed differential AK-*6b* mRNA accumulation during the development of the transformed plants. Massive shoot formation of leaf discs was accompanied by a large accumulation in transcript levels. During later stages of growth some of the abnormal shoots gradually and selectively developed into normal shoots with normal leaves and roots. At this stage whether the plants were grown on MS medium or in soil, mature shoots contained only low levels of AK-*6b* transcripts, although a limited number of transcripts were still recognized in roots of mature plants on MS medium. When leaf discs of mature AK-*6b*-transgenic plants were placed onto MS medium they again produced extensive shoots and repeated the same developmental cycle as above. Therefore, there must be distinct stage-dependent, reversible regulation of AK-*6b* expression: induction, shut-off (silencing), and resetting of the accumulation of transcripts. Transcript accumulation arose from an undifferentiated state of plant cells associated with wounding, since regenerated shoots developed from calli that appeared only from the wounding sites. However, leaf discs that were thought to be extensively wounded did not show rapid accumulation of AK-*6b* transcripts on MS medium. Furthermore, exogenous application of either auxin or cytokinin did not induce AK-*6b* transcript levels. These results indicate that wounding and phytohormones are not by themselves responsible for gene activation. Tinland et al. (1992) noted that the Tm4-*6b* gene product increased wound-induced cell division in *N. rustica*. Based on these observations we postulate that the low levels of AK-*6b* product in the transgenic tobacco responded to the wounding signals, which promotes cell division to generate small calli, and that factors associated with the wound-healing process (presumably in the small

calli) may finally have induced AK-*6b* transcript accumulation.

The AK-*6b* transcript levels were reduced during the normal development of immature shoots. Only a few shoots developed to maturity, suggesting that physiological conditions required for gene silencing were confined to certain tissues within whole, immature shoots. Recently, transgene silencing has been reported in many plant systems, including chitinase (Hart et al., 1992), β-1,3-glucanase (de Carvalho et al., 1992), and *rolB* (Dehio and Schell, 1994) genes. In particular, the *rolB* gene from the T-DNA of the Ri plasmid of *A. rhizogenes* inhibits plant regeneration when the gene was constitutively expressed, and the gene silencing infrequently occurs posttranscriptionally in association with normal development of shoots (Dehio and Schell, 1994). Such developmental regulation of gene silencing resembles the AK-*6b* expression described here and may indicate the common mechanism of gene inactivation despite the fact that these two genes were controlled under different promoters.

Among the transgenic tobacco plants, the K16 and K17 tissues did not show spontaneous maturation of the shoots. These particular tissues contain relatively high levels of AK-*6b* transcripts (Fig. 4), which may not be susceptible to gene inactivation. Such inverse correlation between the transcript levels and shoot maturation is consistent with our view that AK-*6b* is required in the initiation of shoot development but is inhibitory during their maturation. In agreement with this conclusion, Tinland et al. (1992) reported that *N. rustica* plantlets transgenic for the Tm4-*6b* gene under control of a heat-shock promoter formed many shoots with limited development of leaves upon heat treatment. They suggested that the Tm4-*6b* gene promotes shoot formation but is rather inhibitory to leaf development.

Braun and Wood (1976) reported that neoplastic tobacco teratomas generated by the *A. tumefaciens* strain T37 were reversibly suppressed to convert to normal tissues. Presumably, regulated activation and silencing of the T-DNA genes as described here could account for the various phenotypes of crown gall tumors.

Possible Mechanisms of AK-*6b* Action

Initially transformed calli and regenerated calli from leaf discs were mostly shoot forming, and prolonged culture was needed to obtain root formation. Such morphological characteristics are similar to those obtained by expression of the cytokinin biosynthesis gene, the primary action of which is to promote extensive shoot formation and to inhibit root proliferation in the culture medium (Skoog and Miller, 1957). Therefore, AK-*6b* could possess cytokinin-like activity and this activity must be weak under the condition of AK-*6b* transcript levels in the shoot-forming calli. This hypothesis, however, does not preclude the possibility that AK-*6b* also possesses an auxin-like function with an activity that could be even weaker than its cytokinin-like properties in manifesting the shooty phenotype. The weak cytokinin-like activity may explain why, at least in the early stages of shoot development induced by AK-*6b*, shoot proliferation was less abundant and the leaves remained unexpanded compared with shoots induced by the cytokinin gene. Furthermore, this putative weak cytokinin-like effect does not appear to contradict AK-*6b* expression in mature roots; only the "strong" cytokinin gene effect is inhibitory to root formation. There are several conceivable possibilities for the molecular function of AK-*6b* to carry out this activity. First, AK-*6b* could be directly involved in hormone metabolism or, alternatively, it may regulate the endogenous hormone biosynthesis genes of the plant. The resulting imbalance of hormonal levels would then explain the exogenous phytohormone-independent growth and development. However, measurement of intracellular cytokinin levels revealed that, in tissues expressing AK-*6b*, levels were as low as those of wild-type shoots and normal shoots of the transgenic plants. This conclusion is further supported by the finding that the pathogenesis-related type 1 protein gene, which is reported to be induced by cytokinin stress (Memelink et al., 1987), was not induced in the shooty calli of the AK-*6b* transgenics (data not shown). Tinland et al. (1992) provided the evidence that action of the *6b* gene may not be mediated by diffusible products such as phytohormones and they proposed that the *6b* gene product maintains the sensitivity of transformed cells to hormones. Taken together with our finding that the AK-*6b*-containing shooty calli possesses normal levels of cytokinins, it is possible that the AK-*6b* product influences cytokinin signal perception by modulating the cytokinin receptors.

The hormonal status (other than cytokinins) of these AK-*6b* plants remains to be determined. It is unlikely, however, that the auxin levels are increased by AK-*6b*, since excess auxin promotes root formation and AK-*6b* induces shoot formation. GA may be affected, since Nakagawa et al. (1987) showed that the endogenous GA content was correlated with shooty teratoma formation by *A. tumefaciens*.

Interaction of AK-*6b* and the Hormone Synthesis Genes

In an earlier report Tinland et al. (1989) demonstrated that the Tm4-*6b* gene enhanced tumor formation on *N. rustica* induced by the auxin gene of the strain Tm4. The S4-*6b* gene also increased the size of crown galls induced on *N. rustica* by the action of the auxin gene and also by the cytokinin gene. In agreement with these findings, our results indicate that transgenic plants responded to the auxin and cytokinin genes by forming large, unorganized crown gall tumors on intact plant stems. Furthermore, when the tumorous tissues were excised and transferred to MS medium, they showed distinct morphology; the auxin gene-induced tumors developed unorganized calli with infrequent roots, whereas the cytokinin gene-induced tumors developed into shooty calli. The morphological differences in tumors developing on intact plants and on culture medium may have reflected the differential effects of environmental factors from the surrounding tissues of intact stems and those from the MS medium. In either case, AK-*6b* was found to promote the effects of the individual hormone biosynthesis genes on phenotypes of the plants. The auxin-induced tumor morphology could be interpreted such that the weak, cytokinin-like activity of AK-*6b* may complement

the auxin effects and cause relatively unorganized callus formation. Occasional root formation might then have occurred as a result of a dominant effect of the auxin gene over that of AK-*6b*.

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