The EMBO Journal vol.10 no.13 pp.3983 – 3991, 1991

T-DNA gene 5 of *Agrobacterium* modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants

Henrike Körber[1], Nicolai Strizhov[1,2], Dorothee Staiger[1], Joachim Feldwisch[1], Olof Olsson[3], Göran Sandberg[3], Klaus Palme[1], Jeff Schell[1] and Csaba Koncz[1,4,5]

[1]Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-5000 Köln 30, FRG, [2]Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, 142292 Puschino, Moscow Region, USSR, [3]Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S-90183 Umea, Sweden and [4]Institute of Plant Physiology, Biological Research Centre of Hungarian Academy of Sciences, H-6701 Szeged, PO Box 521, Hungary

[5]To whom correspondence should be addressed (Köln)

Communicated by J.Schell

Oncogenes carried by the transferred DNA (T-DNA) of *Agrobacterium* Ti plasmids encode the synthesis of plant growth factors, auxin and cytokinin, and induce tumour development in plants. Other T-DNA genes regulate the tumorous growth in ways that are not yet understood. To determine the function of T-DNA gene 5, its coding region was expressed in *Escherichia coli*. Synthesis of the gene 5 encoded protein (26 kDa) correlated with a 28-fold increase in conversion of tryptophan to indole-3-lactate (ILA), an auxin analogue. Expression of chimeric gene 5 constructs in transgenic tobacco resulted in over-production of ILA that enhanced shoot formation in undifferentiated tissues and increased the tolerance of germinating seedlings to the inhibitory effect of externally supplied auxin. Promoter analysis of gene 5 in plants revealed that its expression was inducible by auxin and confined to the vascular phloem cells. *cis*-regulatory elements required for auxin regulation and phloem specific expression of gene 5 were mapped to a 90 bp promoter region that carried DNA sequence motifs common to several auxin induced plant promoters, as well as a binding site for a nuclear factor, Ax-1. ILA was found to inhibit the auxin induction of the gene 5 promoter and to compete with indole-3-acetic acid (IAA) for *in vitro* binding to purified cellular auxin binding proteins. It is suggested therefore that ILA autoregulates its own synthesis and thereby modulates a number of auxin responses in plants.

Key words: auxin antagonism/auxin regulated gene expression/T-DNA gene 5/indole-3-lactate/plant oncogenes

Introduction

Wounded plants release phenolic compounds that activate the expression of virulence genes located on the chromosome and Ti plasmids of plant pathogenic agrobacteria. In response, agrobacteria attached to plant cell walls mobilize a segment of their Ti plasmid, the T-DNA, which is transferred to plant cells and integrated into the nuclear genome by illegitimate recombination (for reviews see Zambryski, 1988; Zambryski *et al.*, 1989; Mayerhofer *et al.*, 1991). The T-DNA encodes genes that are expressed in plants. Except for genes 5, 6a, 6b and 7, the function of Ti plasmid T-DNA genes is known (for reviews see Nester *et al.*, 1984; Schell, 1986; Binns and Thomashow, 1988). T-DNA genes *iaa*M, *iaa*H and *ipt*Z are termed oncogenes, because they encode the synthesis of plant growth factors, auxin and cytokinin, the overproduction of which results in neoplasmic cell proliferation and appearance of typical crown galls on *Agrobacterium* infected plants (Schröder *et al.*, 1984; Inze *et al.*, 1984; Akiyoshi *et al.*, 1984). It was observed early on that the phenotype of crown galls induced by diverse *Agrobacterium* strains can differ considerably (for review see Schell and Kahl, 1984). Tumour cells carrying the T-DNA of Ti plasmid Ach5 thus develop to compact galls in contrast to cells transformed by the T-DNA of Ti plasmid T37, which differentiate into shoot-forming teratomas. Analogous growth responses with wild type plant tissues can be obtained by modifying the auxin:cytokinin ratios in tissue culture media (Linsmaier and Skoog, 1965). The fact that diverse phenotypes of different crown galls remain stable in growth factor free axenic cultures indicates that T-DNA segments of Ti plasmids are capable of controlling the differentiation of tumour cells, probably by fine tuning of the action of auxin and cytokinin. The level of expression of the T-DNA gene 5 depends on the auxin:cytokinin ratio (Koncz and Schell, 1986) and was found to be relatively high in shoot-forming teratomas, but barely detectable in undifferentiated compact tumours (Joos *et al.*, 1983; Willmitzer *et al.*, 1983). It was conceivable therefore that this T-DNA gene is involved in regulation of the activity of the phytohormones by sensing their concentration and by antagonizing their action. Experiments described in this report support this general view and demonstrate that T-DNA gene 5 modulates auxin response in plants by autoregulated synthesis of an auxin antagonist, indole-3-lactate.

Results

Gene 5 encodes the synthesis of indole-3-lactate

In order to determine the function of T-DNA gene 5, its coding region was expressed in *Escherichia coli*. Gene 5 sequences were dissected as a *Bgl*II–*Eco*RV fragment from plasmid pGV153 which carries a part of the T_L-DNA of Ti plasmid Ach5 (Gielen *et al.*, 1984). A gene cassette extending 28 bp upstream of the ATG initiation codon and 238 bp downstream of the stop codon was constructed by Bal31 exonuclease treatment. This gene cassette was fused to the inducible *tac* promoter of *E.coli* expression vector pTTQ18 (Stark, 1987). An *Rsa*I fragment of gene 5 (positions 1334–1869 of the T_L-DNA, Gielen *et al.*, 1984) was cloned in plasmid pEA305-*Hin*dIII-1 (John *et al.*, 1985) to construct a C-terminal fusion between the truncated

coding region of gene 5 and the cI protein gene of phage lambda. To prepare a fusion between gene 5 protein and protein A from *Staphylococcus*, a *Sal*I gene 5 cassette was constructed by site-specific mutagenesis (Kramer *et al.*, 1984) and cloned in plasmid pRIT2T (Nilsson *et al.*, 1985). Antibody was raised against the gene 5−cI fusion protein, purified with the help of the protein A−gene 5 fusion protein and used for monitoring gene 5 expression in *E.coli* cells carrying plasmid pTTQ18g5 (Figure 1a, see Materials and methods).

To test whether gene 5 was involved in auxin metabolism, bacteria were grown in the presence of L-tryptophan (Trp) or indole-3-acetic acid (IAA). Indole compounds extracted from these bacterial cultures were analysed by TLC. Synthesis of the expected 26 kDa gene 5 protein in Trp treated cells correlated with the accumulation of an indole derivative that comigrated with indole-3-lactate (ILA) in TLC (Figure 2a). The TLC separated compound was further purified by HPLC and shown to be identical with ILA by gas chromatography and mass spectrometry (Figure 1b). Measurement of the conversion of [^{2}H]Trp to [^{2}H]ILA, using selected ion monitoring and [^{13}C]IAA as an internal standard, revealed that gene 5 expression in *E.coli* increased ILA synthesis to 28-fold (from 0.73 to 20.45 × 10^{-6} M).

Phenotypic and physiological effects of ILA production in plants

Although ILA had been previously detected in plants and was regarded to be an auxin analogue with weak hormonal activity (Scott, 1984), its physiological function, if any, has remained obscure. To explore the effect of ILA overproduction in transgenic plants, the coding region of gene 5 was fused to the promoters of cauliflower mosaic virus 35S RNA, mannopine synthase (*mas*) and potato ST-LS1 genes in plant expression vectors pPCV701, 702, 706 and 708 (Koncz *et al.*, 1990; Walden *et al.*, 1990) and transferred into tobacco (see Materials and methods). At least 50 independent primary transformants obtained with each of these vectors, as well as their T2 progenies, were grown in soil in the greenhouse. Plants carrying a low copy number of vector inserts and expressing high levels of gene 5 transcript and protein were selected for further analysis (Figure 3a−c). In accordance with observation of an initial growth reduction, the levels of free IAA were 10−35% lower in transgenic plants than in wild type controls examined at the 5 leaves stage (data not shown). Quantitative measurements using [^{13}C]IAA as an internal standard demonstrated that while IAA and ILA levels in leaves of transgenic plants were similar (97.0 and 107 pmol/g respectively), leaves of wild type plants of the same age contained on average 148 pmol/g IAA and <0.1 pmol/g ILA. These data confirmed that the expression of gene 5 in transgenic plants leads to the synthesis of ILA. Except for a slight and transient reduction of the growth rate of young seedlings, this accumulation of ILA did not appear to influence normal plant growth and differentiation.

Competitive−antagonistic interactions of ILA and auxins

It was noticed that transgenic tobacco calli expressing gene 5 regenerated shoots earlier than control wild type calli on appropriate culture media. This observation suggested that ILA might antagonize auxin action, since alteration of auxin: cytokinin ratios in favour of cytokinin is known to promote

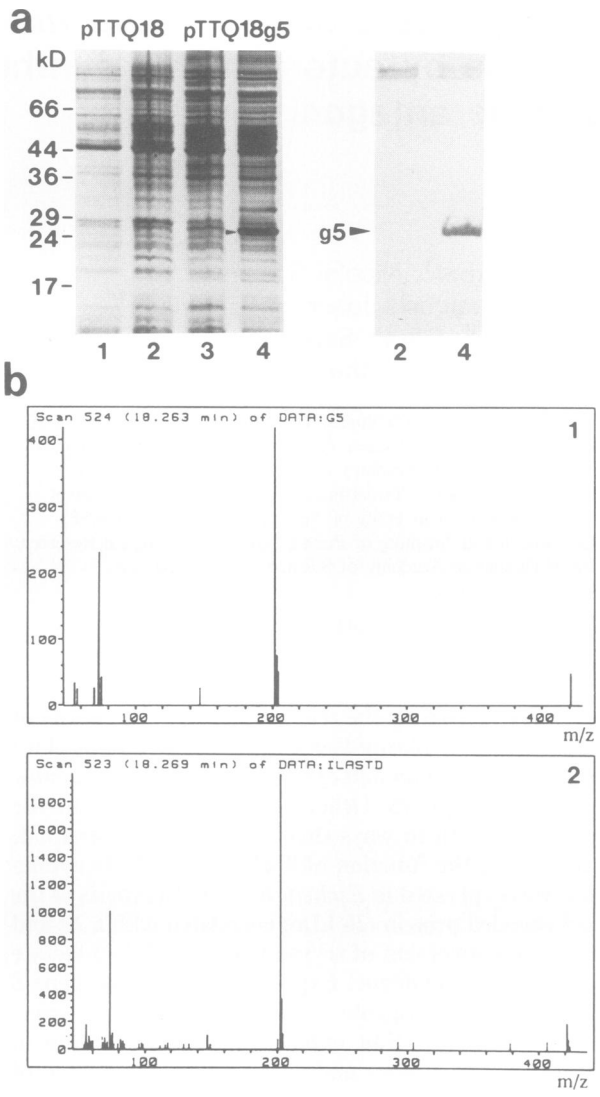

Fig. 1. (**a**) Expression of T-DNA gene 5 in *E.coli*. Protein extracts were prepared from *E.coli* strains carrying the control plasmid pTTQ18 (lanes 1 and 2) and gene 5 expression vector pTTQ18g5 (lanes 3 and 4) before (lanes 1 and 3) or after (lanes 2 and 4) induction of the *tac* promoter by IPTG (Stark, 1987) and separated on a 12.5% SDS−PAA gel. Proteins in lanes 2 and 4 were immunoblotted using antibody raised against gene 5−cI fusion protein and treated with an alkaline phosphatase conjugated goat anti-rabbit antibody. Position of the 26 kDa protein encoded by gene 5 is indicated by an arrow. (**b**) Identification of the indole compound detected by TLC in gene 5-expressing bacteria after feeding with Trp. The TLC separated compound was purified by HPLC and gas chromatography and analysed by mass spectrometry. A plot of mass/charge (*m/z*) ratios obtained for this compound (1) is compared with that of ILA standard (2).

shoot formation by tobacco calli (Linsmaier and Skoog, 1965). The fact that an external supply of auxins can inhibit the growth of tobacco seedlings was used in a biological assay to test auxin antagonistic effect of ILA. Tobacco seeds from wild type and gene 5 transformed plants were germinated on media containing 0.5−2.5 × 10^{-6} M NAA in combination with various cytokinins (0.4 × 10^{-6} M), such as N^6-(2-isopentenyl)adenosine (iPA) or 6-benzylaminopurine (BAP) or 6-furfurylaminopurine (kinetin). Wild type seedlings were retarded when germinated on these media. In contrast, on the hypocotyl of seedlings expressing gene 5, calli appeared and developed to teratoma-like tissues

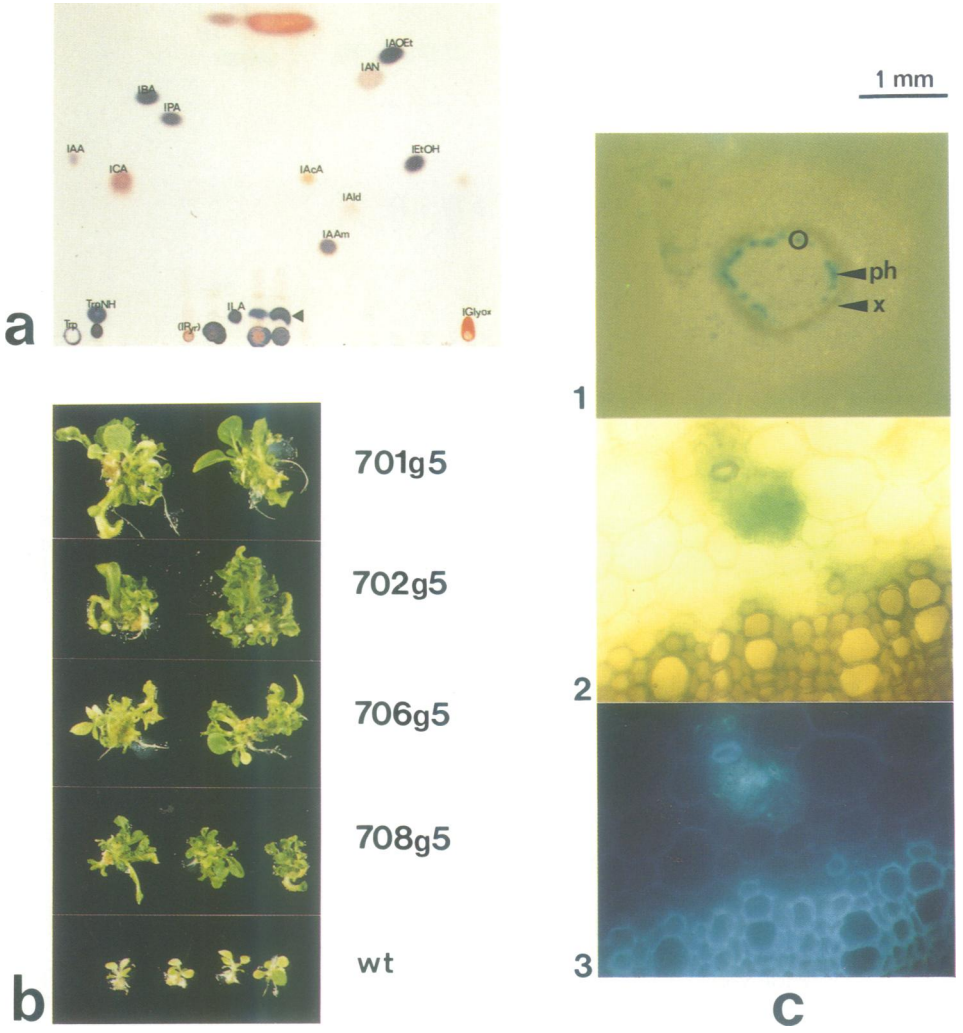

Fig. 2. TLC of indole compounds. *E.coli* strains carrying control plasmid pTTQ18 and gene 5 expression vector pTTQ18g5 were grown in the presence of 0.2 mg/l Trp at 28°C for 48 h. Indole compounds extracted from culture filtrates were separated on Kieselgel F254 plates in a solvent $CHCl_3$:CH_3COOH (95:5). 1: Extract from pTTQ18 transformed cell cultures; 2: ILA standard; 3: extract from gene 5 expressing bacterial cultures; 4: extract 3 mixed with ILA standard. Indole derivatives used as standards were Trp, IAA, tryptamine (TrpNH), indole-3-carboxylic acid (ICA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), indole-3-pyruvate (IPyr), ILA, indole-3-acrylic acid (IAcA), indole-3-acetamide (IAAm), indole-3-aldehyde (IAld), indole-3-acetonitrile (IAN), indole-3-acetic acid ethyl ester (IAOEt), indole-3-ethanol (IEtOH) and indole-3-glyoxylic acid (IGlyox). Position of ILA is marked by an arrow. (**b**) Teratoma shoot formation of transgenic tobacco seedlings carrying the coding region of gene 5 in plant expression vectors pPCV701 (701g5), pPCV702 (702g5), pPCV706 (706g5) and pPCV708 (708g5). Seeds derived from the T2 generation of transgenic plants were germinated on LS medium (Linsmaier and Skoog, 1965) containing 0.5×10^{-6} M NAA, 0.4×10^{-6} M BAP and 100 mg/l kanamycin. Wild type (wt) control plants were germinated on the same medium without kanamycin. (**c**) 1: Histological staining of a stem cross-section from a transgenic tobacco plant that was transformed with a construct carrying the −128 to −292 region of gene 5 promoter upstream of the TATA box minimal promoter of T-DNA gene 2 and β-glucuronidase reporter gene in vector pGDW4422. Area marked by a circle is enlarged in section 2 and showed after staining of callose cell walls by aniline dye blue in section 3. ph: phloem.; x: xylem.

with multiple shoots (Figure 2b). When calli derived from ILA-producing transgenic plants were grown on a medium containing 0.5×10^{-6} M NAA and 0.4×10^{-6} M BAP, they formed shoots, whereas wild type calli grew as undifferentiated tissues. ILA addition to callus media also enhanced shoot formation by wild type calli. The degree to which ILA was capable of overcoming the inhibitory effect of auxin (NAA) in these seed germination and shoot induction assays appeared to depend to some extent on the type of cytokinin applied (data not shown).

Interestingly, when tobacco plants were tested on media containing cytokinin or NAA and cytokinin in combination with ILA (up to 1×10^{-4} M), no difference in development of wild type and transgenic plants was observed. Different responses of plants, germinating seedlings and undifferentiated callus tissues to ILA, supplied in culture media or synthesized in transgenic plants, indicated that auxin antagonistic effect of ILA may substantially differ by the type and differentiation stage of target plant tissues. These observations also suggested that the capability of ILA to act as auxin antagonist could be due to a competition between ILA and auxins for binding to specific cellular auxin receptors or carriers.

To test this hypothesis, interaction of ILA with diverse cellular auxin binding proteins (ABPs) was examined using *in vitro* photoaffinity auxin label, 5-azido-[7-^{3}H]IAA ([^{3}H]-N_3IAA) in auxin competition assays. ABPs were prepared from maize coleoptiles, incubated with [^{3}H]N_3IAA in the presence of an excess of unlabelled IAA, NAA or ILA, illuminated to cross-link the photoaffinity label and analysed by gel electrophoresis. ILA and NAA competed with the IAA label for binding to distinct classes of ABPs. Among

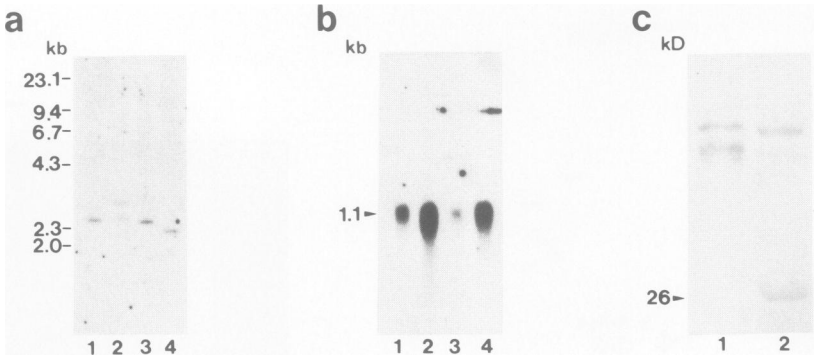

Fig. 3. Expression of chimeric gene 5 constructs in transgenic tobacco. (**a**) Southern DNA hybridization of *Eco*RI digested nuclear DNAs prepared from transgenic tobaccos carrying gene 5 expression vectors pPCV701g5 (lane 1), 702g5 (lane 2), 706g5 (lane 3) and 708g5 (lane 4) with the coding sequence of the *aph*(3′)II gene as probe. *aph*(3′)II sequences were parts of plant selectable marker genes located in all vectors close to the right end of the T-DNA (Koncz *et al.*, 1990). Therefore the probe detected genomic DNA fragments corresponding to the right plant DNA junction fragment of T-DNA inserts. (**b**) 10 μg poly(A)$^+$ RNA prepared from transgenic tobacco plants 701g5 (lane 1), 702g5 (lane 2), 706g5 (lane 3) and 708g5 (lane 4) characterized in (a) was hybridized on Northern filters with the *Bgl*II−*Bam*HI fragment of pKC7g5 DNA, which contained the coding region of gene 5 as probe. Position of gene 5 transcript is marked by an arrow. (**c**) Detection of gene 5 protein in a transgenic 706g5 tobacco plant. Leaf proteins were isolated from 0.3 mg tissue samples of wild type (lane 1) and transgenic plants (lane 2), separated on a 12.5% SDS−PAA gel, blotted to nitrocellulose filters and treated with antibody against gene 5−cI fusion protein, then with a ^{35}S-labelled donkey anti-rabbit IgG. Arrow points to the position of the 26 kDa gene 5 protein.

these were ABP1, a protein located in the endoplasmic reticulum (Hesse *et al.*, 1989), as well as pm23 and pm24, which are plasma membrane-bound ABPs thought to be involved in cellular auxin transport (Figure 4; Palme *et al.*, 1991).

ILA autoregulates the auxin induced expression of gene 5

Preliminary analysis of the gene 5 promoter indicated that gene 5 expression in callus tissues was regulated by the ratio of auxin to cytokinin, whereas in transgenic plants it followed a basipetal gradient in stem and petioles (Koncz and Schell, 1986). To determine whether spatial patterns of gene 5 expression resulted from a transcriptional response to diverse cellular auxin levels, the tissue specificity and auxin activation of reporter gene constructs fused to various segments of the gene 5 promoter were tested. The promoter of gene 5 was dissected as a *Cla*I−*Bgl*II fragment from plasmid pPCV002 (positions 605−1016 of the T_L-DNA; Koncz and Schell, 1986) and further cleaved by *Alu*I, *Hin*cII, *Fsp*I, *Ava*I and *Hin*fI enzymes (Figure 5a). Promoter fragments representing nested deletions from and towards the *Bgl*II site (located at position −40 upstream of the ATG codon) were fused to the TATA box minimal promoter of T-DNA gene 2 driving an *aph*(3′)II reporter gene in the enhancer test vector pGDW4411 (Wing *et al.*, 1989; Walden *et al.*, 1990). In addition, fragments derived from promoter region −128 to −352 were cloned in vector pGDW4411 to obtain fusions with a defined spacing between the heterologous TATA box and various upstream elements of the gene 5 promoter. After transformation of tobacco, five transgenic plants for each of these constructs were pooled and the expression of the reporter gene was analysed in particular for tissue specificity and inducibility by auxin. Plants transformed with vector pGDW4411 without any insert were used as control. To test for induction by auxin, protoplasts were prepared from leaves, treated for 12 h either with 0.4×10^{-6} M BAP alone, or with 0.5×10^{-6} M NAA and 0.4×10^{-6} M BAP, and assayed for aph(3′)II enzyme activity. Enzyme activities measured in NAA- and BAP-treated protoplasts were compared with samples treated

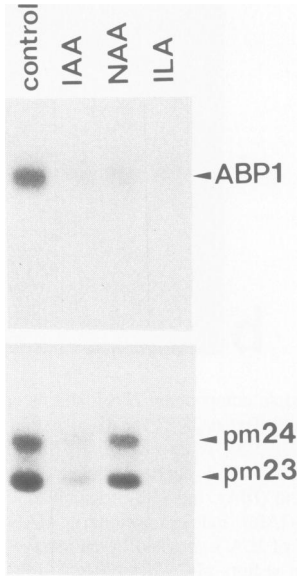

Fig. 4. *In vitro* auxin competition assay with purified ABPs. Purified ABP1, pm23 and pm24 proteins were incubated with 0.1 μM [^{3}H]N_3IAA photoaffinity label in the presence of 0.1 mM IAA, NAA or ILA, illuminated, separated on a 12.5% SDS−PAA gel and visualized by autoradiography.

with BAP only, as well as with similar samples of protoplasts harbouring the reporter gene driven only by the minimal promoter of vector pGDW4411. Distribution of reporter enzyme activity in leaf, petiole, stem and root tissues of the same plants was also determined and compared with data obtained in protoplast assays (Figure 5b). The analysis demonstrated that the shortest fragment, which directed gene expression only in stem and petioles and allowed expression in protoplasts after induction with auxin, was located within the −202 to −292 region of the promoter. DNA sequences flanking this region from position −128 to −352 did not alter the specificity, but increased the activity of recombinant gene 5 promoters (data not shown). A correlation between auxin response and tissue specific expression was also observed in histological studies. A promoter region from

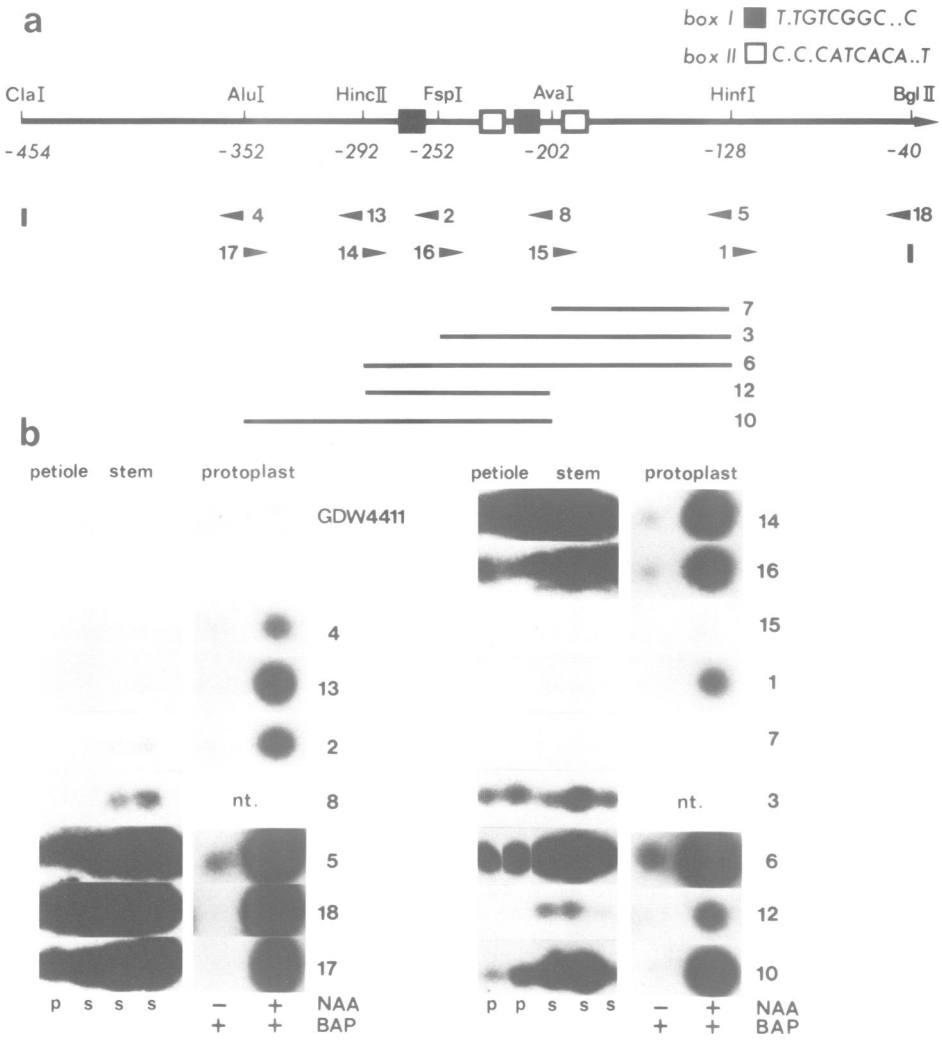

Fig. 5. Analysis of *cis*-regulatory elements in the promoter region of gene 5. (**a**) Schematic map of the promoter region. Direct repeats were designated as boxI and boxII. Endonuclease cleavage sites used for generation of deletions are indicated. Numbers and arrows below the map show the position of promoter fragments which were cloned in the enhancer test vector pGDW4411. (Thus construct 4 corresponds to promoter region −454 to −352, construct 1 carries the region −128 to −40, etc.) (**b**) Assay of tissue specific activity and auxin induction of various gene 5 promoter constructs. aph(3′)II reporter enzyme activity was assayed in leaves, petioles (p), stem (s) and roots of transgenic plants carrying different gene 5 promoter constructs identified by numbers, or vector pGDW4411 alone, as control. aph(3′)II activity was not detected in leaves and roots, therefore corresponding data were omitted from the figure. Petiole and stem samples were taken at the five leaves stage from a region between the first and third internodes in the basipetal direction. To test induction by auxin, leaf protoplasts were prepared from each of these plants (five individuals for each promoter construct tested) and treated for 12 h with cytokinin (BAP) alone or with auxin (NAA) and cytokinin (BAP), as described in the text. nt, not tested.

positions −128 to −292 was fused to the minimal promoter of T-DNA gene 2 located upstream of a β-glucuronidase (*gus*) reporter gene in vector pGWD4422 (D.Wing, unpublished; Walden *et al.*, 1990) and transformed in tobacco. Expression of this reporter gene construct increased in a basipetal direction in stem and petioles of transgenic plants (data not shown). Histological staining revealed that *gus* gene expression was confined to vascular protophloem cells (Figure 2c) known to function in auxin transport (Davies, 1987).

Nucleotide sequences within the promoter region from −202 to −292 contained characteristic direct repeats of TGTCGGC and CATCACA motifs, that were designated boxI and boxII (Figures 5a and 6a). Using boxI or boxII as fixed positions in DNA sequence alignments, a significant homology was observed between this putative gene 5 enhancer domain and upstream regulatory sequences of auxin induced soybean *aux*28 and SAUR genes (Ainley *et al.*, 1988; McClure *et al.*, 1989) and *Arabidopsis dbp, aux*2-11 and *aux*2-27 genes (Alliotte *et al.*, 1989; Conner *et al.*, 1990; Figure 6a).

To study interaction of this gene 5 promoter region with nuclear proteins prepared from tobacco seedlings, a *Fsp*I−*Hin*fI promoter fragment (−128 to −252) was used as a probe in band mobility shift assays. A complex obtained with this probe was specifically competed with the unlabelled fragment (Figure 6b, left panel). To map DNA−protein interactions within the promoter region −202 to −252 more accurately, oligonucleotides boxI, II, I+II and boxIII (Figure 6a, see Materials and methods) overlapping the conserved DNA sequence elements were used as probes. In addition, an oligonucleotide, boxI*+II, carrying mutations in boxI, was included as a probe in the assay (Figure 6b, right panels, lanes 1−8). BoxII and boxIII probes formed complexes with tobacco nuclear proteins. Mutations in boxI did not influence protein binding to the boxI*+II probe, indicating that boxII

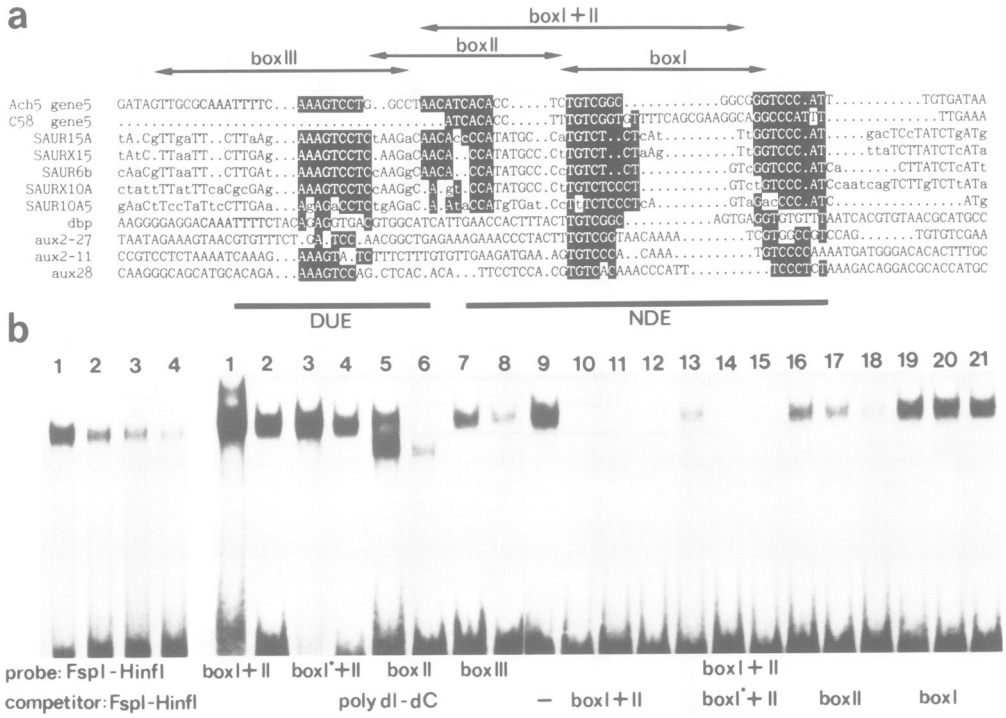

Fig. 6. Comparison of DNA sequence elements conserved between the promoter of gene 5 and promoters of a number of auxin regulated plant genes. The −202 to −252 region of gene 5 promoter carries DNA sequence motifs common to several auxin regulated plant promoters. The DNA sequence alignment shows the −260 to −188 region of the promoter of gene 5 from the T_L-DNA of Ti plasmid Ach5 (Gielen *et al.*, 1984), a part of the corresponding homologous region from the T-DNA of Ti plasmid C58 (M.Lemmers, University of Gent, unpublished), the DUE and NDE conserved promoter regions of soybean SAUR genes as defined by McClure *et al.* (1989), the −264 to −172 promoter region of the *Arabidopsi dbp* gene (Alliotte *et al.*, 1989), the −278 to −190 upstream region of *aux*2-27 and the −252 to −162 region of *aux*2-11 promoters from *Arabidopsis* (Conner *et al.*, 1990), as well as the −300 to −230 promoter region of soybean *aux*28 gene (Ainley *et al.*, 1988). DNA sequences identical in at least three promoters are printed in black. To indicate diverse spacing of conserved sequence elements, dots were introduced in the aligned DNA sequences. The exact sequence of oligonucleotide probes boxI, II, III and boxI+II can be read by ignoring the dots, as described in Materials and methods. Grey background marks a region that was found only in gene 5 and *dbp* promoters. (**b**) Identification of a binding site for a nuclear factor in the boxII region of the promoter of gene 5. Left panel: the *Fsp*I−*Hin*fI fragment (positions −128 to −252) of the promoter was labelled as a probe (0.5 ng/reaction) and incubated with crude nuclear protein extract (7 μg/assay) prepared from tobacco seedlings in the presence of poly(dI−dC) (1 μg/assay) and of increasing amounts of the unlabelled homologous fragment, as competitor. Lane 1: no competitor; lane 2: 50 ng; lane 3: 100 ng; and lane 4: 150 ng of unlabelled *Fsp*I−*Hin*fI competitor fragment. Right panel: lanes 1−8: oligonucleotide probes boxI+II, boxI*+II, boxII and boxIII (0.5 ng of each) were incubated with tobacco nuclear protein extract (8 μg/assay) in the presence of 2 μg (lanes 1, 3, 5 and 7) and 3 μg (lanes 2, 4, 6 and 8) of poly(dI−dC). Lanes 9−21: boxI+II probe (0.5 ng) was incubated with nuclear protein extract (7 μg) in the presence of poly(dI−dC) (3 μg) with unlabelled boxI−II (lanes 9−12), boxI*+II (lanes 13−15), boxII (lanes 16−18) and boxI (lanes 19−21) oligonucleotides as specific competitors. In comparison with TGTCGGC sequences of boxI, boxI*+II oligonucleotide carried a mutated TGTCAAC sequence. In lane 9, no competitor; in lanes 10, 13, 16 and 19, 15 ng; in lanes 11, 14, 17 and 20, 50 ng; and in lanes 12, 15, 18 and 21, 100 ng competitor was added. Other conditions were according to Staiger *et al.* (1989).

sequences were probably required for the binding activity. To test whether boxII was specifically binding a nuclear protein, boxI+II oligonucleotide was used as a probe in combination with unlabelled boxI, II, I+II and I*+II oligonucleotides as competitors. While boxI did not compete with probe, efficient competition was observed with boxII, I+II and I*+II oligonucleotides (Figure 6b, lanes 9−21). This result indicated that it was boxII that contained a binding site for a nuclear factor, designated Ax-1. A correlation between the specific enhancer activity of the −202 to −292 promoter region and the binding of Ax-1 to boxII sequences remains to be determined.

To analyse the time course of auxin induction, leaf protoplasts were prepared from tobacco plants transformed with gene 5 enhancer construct 12 (Figure 5a), which carried the promoter region −202 to −292. The protoplasts were treated with 0.5×10^{-6} M NAA in the presence of BAP (0.4×10^{-6} M). Aliquots taken at different time intervals were assayed for aph(3′)II enzyme activity. The response to auxin as measured by reporter enzyme activity was slow: aph(3′)II activities were detected only 9−12 h after auxin addition (Figure 7a). A similarly slow response to auxin was also observed with another T-DNA derived auxin regulated promoter, that of the *rol*B gene (data not shown, see Maurel *et al.*, 1990). Treatment of protoplasts with $0.5-100 \times 10^{-6}$ M concentrations of NAA, IAA or ILA showed that final levels of aph(3′)II reporter enzyme activity depended on both the type and the concentration of auxin that was used for induction (Figure 7b and c). ILA in combination with BAP induced the expression of the reporter gene. However, increasing concentrations of ILA ($0.5-15 \times 10^{-6}$ M) in combination with NAA (0.5×10^{-6} M) and BAP (0.4×10^{-6} M) gradually inhibited the auxin induced expression of the reporter gene construct (Figure 7d). A 100-fold excess of ILA over NAA completely abolished *aph*(3′)II gene expression. These data showed that the −202 to −292 region of the promoter was involved in autoregulation of the expression of gene 5 and indicated that ILA can, by feedback, regulate its own synthesis antagonizing the induction of gene 5 expression by auxin.

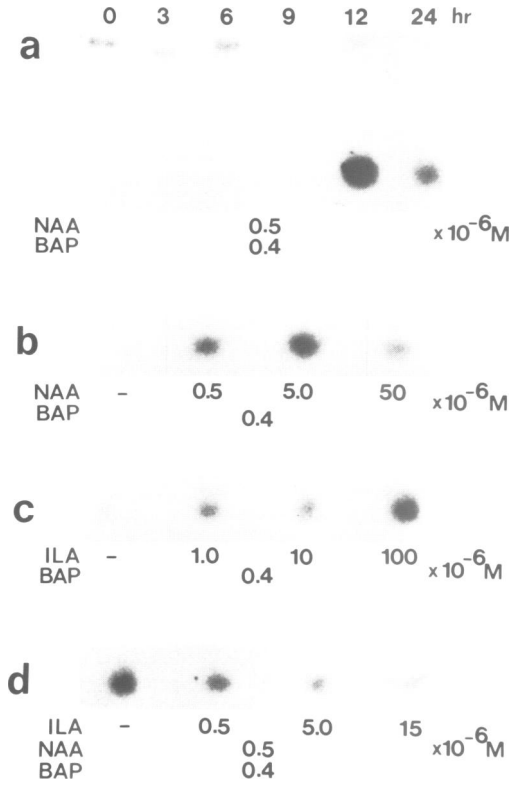

Fig. 7. ILA negatively regulates the induction of gene 5 promoter by auxin. (**a**) Leaf protoplasts prepared from transgenic plants carrying the −202 to −292 region of the promoter of gene 5 in enhancer test vector pGDW4411 were incubated with 0.5×10^{-6} M NAA and 0.4×10^{-6} M BAP. Aliquots containing 10^5 cells/ml were taken at 0, 3, 6, 9, 12 and 24 h and assayed for aph(3′)II enzyme activity. (**b**) Aph(3′)II assay of protoplast samples after incubation for 24 h with 0.5, 5.0 and 50×10^{-6} M NAA in the presence of 0.4×10^{-6} M BAP. (**c**) Protoplasts were incubated for 24 h in the presence of 0.4×10^{-6} M BAP with increasing concentrations (1.0, 10 and 100×10^{-6} M) of ILA before *aph*(3′)II assays. (**d**) Protoplasts were treated for 24 h with increasing concentrations (0.5, 5.0 and 15×10^{-6} M) of ILA in the presence of 0.5×10^{-6} M NAA and 0.4×10^{-6} M BAP.

Discussion

Current models of plant hormone action suggest that cellular activity of auxins is primarily regulated by the sensitivity of plant cells to this growth factor (Trewavas, 1980, 1981; Davies, 1987). Crown gall tumour cells actively produce plant growth factors, auxin and cytokinin, that could cause cell death if they accumulated at high levels. Tumour tissues in axenic cultures, however, proliferate indefinitely. It is logical therefore to assume that in addition to oncogenes involved in the synthesis of phytohormones, the T-DNA must encode other genes for the regulation of the action of auxin and cytokinin in tumour tissues. This assumption, together with the observation that increased expression of the T-DNA gene 5 correlated with differentiation of crown galls to shoot-forming teratomas (Joos *et al.*, 1983; Willmitzer *et al.*, 1983), served as a starting point for the study described above. Since the expression of the T-DNA gene 5 was increased by increasing the auxin:cytokinin ratio (Koncz and Schell, 1986), we assumed that it could play a regulatory role in fine tuning the activity of auxins.

To test this hypothesis, the function of gene 5 was determined after expression of its coding region in *E.coli*. To explore whether the protein encoded by gene 5 was involved in auxin metabolism, the fate of tryptophan was followed in bacteria expressing gene 5. Synthesis of a 26 kDa protein from gene 5 correlated with a 28-fold accumulation of ILA, an auxin analogue. The fact that ILA synthesis was also detected in control, untransformed *E. coli* cultures prompted us to search for data discussing the possible function of ILA in bacteria. This led us to intriguing early reports demonstrating that ILA is produced by several soil bacteria, including the plant pathogenic *Agrobacterium*, as well as symbiotic nitrogen-fixing bacteria, such as *Rhizobium* and *Azospirillum* (Kaper and Veldstra, 1958; Phelps and Sequeira, 1968; Trinchant and Rigaud, 1977; Badenoch-Jones *et al.*, 1982; Zimmer *et al.*, 1988). Some of these reports considered ILA to be a metabolic side-product (Rovenska *et al.*, 1982). In contrast, others suggested that ILA may have an important function, since IAA and ILA synthesis in bacteria is stringently regulated by available oxygen (Trinchant and Rigaud, 1977). Rhizobia thus produce IAA in the free-living state, while as bacteroids in plant nodules, they synthesize only ILA. In retrospect, and in the light of the data presented here, it is tempting to speculate that some soil bacteria may control the growth of roots of host plants by the regulated production of both the active growth factor, IAA, and of its antagonist, ILA. In so doing, these might mimic a similar mechanism operative in the plants themselves. Sembder *et al.* (1980) suggested that indole-3-pyruvate (IPy) is an intermediate for the synthesis of both IAA and ILA. In contrast, Phelps and Sequeira (1968) predicted that the conversion of Trp to ILA occurs in a single step. Whether the protein encoded by gene 5 catalyses a slow direct conversion of Trp to ILA or uses IPy as substrate remains to be determined.

ILA has also been detected in plants (Wightman, 1973) but, as in bacteria, its physiological role was unclear. To explore the effect of cellular ILA production in plants, T-DNA gene 5 was expressed in transgenic tobacco using diverse plant promoters. Interestingly, neither intracellular production nor external supply of ILA markedly influenced the differentiation of plant organs. However, a defined phenotype was observed when undifferentiated callus tissues were derived from leaves of transgenic plants expressing gene 5. Cellular synthesis of ILA in calli or the presence of ILA in the medium facilitated the differentiation of shoots. This observation suggested that gene 5-mediated synthesis of ILA in crown gall tumours might well be responsible for the formation of teratoma shoots (Joos *et al.*, 1983). A second consequence of gene 5 expression in transgenic tobacco plants was their capacity to tolerate external concentrations of auxin, such as NAA, which inhibited the growth of non-transformed wild type plants. Since neither IAA nor NAA are substrates for the gene 5 protein, the anti-auxin effect of ILA observed here indicated that ILA probably competes with some cellular auxin functions.

Efficient *in vitro* competition of ILA with IAA for binding to putative auxin carrier and transport proteins, such as ABP1, pm23 and pm24 (Hesse *et al.*, 1989; Palme *et al.*, 1991), in fact supports this notion. This competition for binding to cellular proteins involved in the transduction of auxin signals to nuclear target genes suggests that ILA can probably antagonize auxin stimulation of gene expression in tumours, as well as in normal plant cells. Analysis of

the regulation of gene 5 promoter activity indicates that this is probably also the mechanism by which ILA negatively controls its own synthesis.

Indeed, the activity of the promoter of gene 5 was found to be dependent on auxin. Fusion of *cis*-regulatory sequences from gene 5 promoter to the TATA box region of a heterologous minimal promoter allowed us to identify a 90 bp enhancer region that is sufficient to regulate auxin induced reporter gene expression in protoplasts and confines gene expression to differentiating phloem cells in stem and petioles. These tissues are thought to play an important role in auxin transport (Scott, 1984; Davies, 1987). It is particularly intriguing that this putative enhancer region of gene 5 promoter carries DNA sequence elements that are common to *cis*-regulatory regions in several auxin regulated plant genes. Identification of a nuclear factor, Ax-1, which binds specifically to this region, provides a possibility for further exploration of the connections between promoter regulation and auxin signal transduction.

Consistent with the idea that ILA antagonizes auxin stimulated gene expression by competing for binding to elements of the auxin signal transduction chain is the observation that whereas ILA itself is capable of stimulating the activity of the promoter of gene 5, it caused repression of auxin induced expression of reporter gene constructs. This autoregulatory effect of ILA on its own synthesis most likely reflects a more general mechanism, as explained above.

In summary, the analysis of the function and regulation of gene 5 illustrates the remarkable elegance and efficient genetic make-up of the transferable T-DNA element of *Agrobacterium* Ti plasmids. This T-DNA not only encodes oncogenes that stimulate tumorous development by the production of major plant growth factors, auxin and cytokinin, but also a function, determined by gene 5, to modulate the effects of auxin. Gene 5 overcomes toxicity of an auxin overdose by providing for the autoregulated synthesis of ILA, which acts as an antagonist to auxin by competing for binding to proteins involved in auxin signal transduction. We expect that another T-DNA gene, gene 6b (Spanier *et al.*, 1989; Tinland *et al.*, 1989), might have a similar function in modulating cytokinin activity. Finally, it is also tempting to speculate that this mode of fine tuning of growth factor activity (i.e. competition between activator ligand and analogous antagonist for binding to receptors) is not limited to T-DNA genes, but might reflect a general mechanism in plants.

Materials and methods

Expression of T-DNA gene 5 in E.coli

Recombinant DNA techniques, protein analysis, antibody production and purification and immunological techniques were according to Sambrook *et al.* (1989) and Harlow and Lane (1988) unless stated otherwise. The coding region of gene 5 was excised as a *Bgl*II–*Eco*RV fragment from plasmid pGV153 (Gielen *et al.*, 1984; T_L-DNA positions 1020–2105). After conversion of the *Eco*RV site to a *Bam*HI site by linker addition, this fragment was subcloned in plasmid pKC7g5 (Rao and Rogers, 1979). The *Bgl*II–*Bam*HI fragment from pKC7g5 DNA was treated by Bal31 exonuclease, subcloned into the *Sma*I site of M13 phage vector mp19, characterized by DNA sequencing (Yanisch-Perron *et al.*, 1985; Tabor and Richardson, 1987), excised from mp19 and cloned as an *Eco*RI–*Bam*HI fragment in expression vector pTTQ18g5. This *Eco*RI–*Bam*HI fragment was also inserted into vector mp9 *am* and annealed to the oligonucleotide primer (5′-CCGTCACTTAAG*TCGAC*ATGTATGAGCGG-3′) and mp9 *rev* DNAs to generate a *Sal*I site (labelled by asterisks) upstream of the ATG codon (underlined) by site-specific mutagenesis (Kramer *et al.*, 1984). The constructed gene cassette was cloned into the *Sal*I site of plasmid pRIT2T (Pharmacia) to produce a gene 5–protein A fusion (Nilsson *et al.*, 1985). An *Rsa*I fragment of gene 5 (T_L-DNA positions 1334–1860; Gielen *et al.*, 1984) isolated from pKC7g5 DNA was cloned into the filled-in *Hin*dIII site of plasmid pEA305-*Hin*dIII-1 (John *et al.*, 1985) to prepare a gene 5 protein fusion to phage lambda cI protein. This cI–gene 5 fusion protein (32 kDa) was purified from SDS–polyacrylamide (PAA) gels and used for raising antibody in rabbit. The crude antibody was purified with the help of protein A–gene 5 fusion protein that was immobilized on a CNBr-activated Sepharose matrix (Pharmacia). Expression of gene 5 carried by vector pTTQ18 was induced at 28°C for 48 h or at 37°C for 2.5 h as described (Stark, 1987). Bacteria were cultured in minimal A medium supplemented with 0.2 mg/l of either Trp, IAA, NAA, ILA, IPy or with [^{2}H]Trp and [^{13}C]IAA, as internal standard. Synthesis of the 26 kDa gene 5 protein was monitored by immunoblotting. Indole compounds were extracted from bacterial cultures and analysed by TLC, HPLC, gas chromatography and mass spectrometry as described (Stahl, 1967; Sandberg *et al.*, 1987; Sembder *et al.*, 1988). For determination of auxin content, leaf and stem segments were collected and pooled from the third and fifth internode regions of three to 10 transgenic or wild type plants of similar age. Extraction, qualitative and quantitative analysis of auxins in plant samples were according to Sandberg *et al.* (1990). Data from at least three independent measurements were combined.

Tissue culture and transgenic plant technology

Coding region of gene 5 was excised as a *Bgl*II–*Bam*HI fragment from pKC7g5 DNA, cloned in the *Bam*HI site of the plant expression vectors pPCV701, 702, 706 and 708 (Koncz *et al.*, 1990) and transferred into *Agrobacterium* strain GV3101 (pMP90RK) (Koncz and Schell, 1986) to transform *Nicotiana tabacum* SR1. All methods, media used for induction of calli and shoots, protoplast isolation and seed germination were as described (Koncz and Schell, 1986; Spena *et al.*, 1987; Koncz *et al.*, 1989). Seedlings from the T2 generation of transgenic plants were germinated in the presence of 100 mg/l kanamycin and transferred to soil for measurement of growth rate and auxin content. Phenotypic effects of ILA were tested as described in Results.

In vitro auxin competition assays with purified ABPs

Preparation of ABP1, pm23 and pm24 was according to Hesse *et al.* (1989) and Feldwisch *et al.* (1991). For photoaffinity labelling, proteins were incubated with 1 μM [^{3}H]N_3IAA (a kind gift from N.Campos) in buffer [10 mM Tris–MES (pH 6.5) and 0.25 M sucrose] in the presence of 0.1 mM unlabelled competitor, such as IAA, NAA or ILA. After photolysis at −196°C for 10 min, proteins were precipitated, separated by 12.5% SDS–PAGE and analysed by autoradiography.

Analysis of gene 5 expression and promoter constructs in transgenic plants

DNA and RNA purifications, electrophoresis, blotting and hybridization, protein extraction from plant tissues and immunoblotting were as described (Koncz and Schell, 1986; Harlow and Lane, 1988; Koncz *et al.*, 1989, 1990; Sambrook *et al.*, 1989). The enhancer test vector pGDW4411 was obtained by deletion of a *Cla*I–*Bgl*II fragment from plasmid pGDW44 (Wing *et al.*, 1989). Vector pGDW4422 (D.Wing, unpublished) has been reported by Walden *et al.* (1990). The promoter of gene 5 (T_L-DNA positions 605–1916; Gielen *et al.*, 1984) was excised from plasmid pPCV002 (Koncz and Schell, 1986) as a *Cla*I–*Bgl*II fragment and further digested with *Alu*I, *Hin*cII, *Fsp*I, *Ava*II or *Hin*fI enzymes. Promoter fragments were inserted into the *Hin*cII site of M13 vector mp7, then released with *Eco*RI and cloned into the *Eco*RI sites of vectors pGDW4411 and pGDW4422 in the same orientation as they occurred within the gene 5 promoter. All promoter fusions were verified by DNA sequencing and transferred using *Agrobacterium* into tobacco. Quantitative assay of aph(3′)II reporter enzyme was as described (Reiss *et al.*, 1984; Koncz and Schell, 1986), except that it was standardized for an equal number of cells when protoplasts were used in auxin induction assays. Fluorimetric measurement and histological staining of β-glucuronidase activity was as described (Jefferson, 1987). Callose cell walls were counter-stained with aniline blue according to Braune *et al.* (1983).

Nuclear protein extracts were isolated from tobacco tissues and used in band shift gel assays as described (Staiger *et al.*, 1989). Conditions used in various experiments are specified in the Figure 6 legend. Double-stranded oligonucleotides applied as specific probes and competitors were boxI (5′-aattcggatccCTGTCGGCGGCGGGgatcc-3′), boxII (5′-aattcgga-TGGCCTAACATCACACCTCatccg-3′), boxI+II (5′-aattcCTAA-CATCACACCTCTGTCGGCGGCg-3′), boxI*+II (5′-aattcCTAACAT-CACACCTCTGTCA*A*CGGCg-3′, asterisks mark the mutated positions), and boxIII (5′-gatccAGTTGCGCAAATTTCAAAGTCCTGGC-3′), where capital letters denote gene 5 promoter sequences. Oligonucleotides were

subcloned as monomers in pUC vectors (Yanisch-Perron *et al.*, 1985) to determine their nucleotide sequence. For nucleotide sequence comparison a program package adapted to Vax/VMS computer version 5.1-1 was exploited (Devereux *et al.*, 1984).

Acknowledgements

We thank A.Radermacher and A.Lossow for skilful technical assistance, K.Pawlowski for help in DNA sequence analysis, D.Wing, M.John and J.Schmidt for kindly providing plasmid vectors, I.Moore for stimulating discussion and help in enzyme assays, A.Östin for help in GC–MS analysis, G.Kobert for preparation and R.Walden for critical reading of the manuscript. This work was supported as part of a joint project between the MPI (Köln) and BRC (Szeged) by the Deutsche Forschungsgemeinschaft and the Hungarian Academy of Sciences as well as by the Swedish Council for Forestry and Agricultural Research (SJFR), and is part of a collaborative project between the MPI (Köln) and the Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences (Puschino).

References

Ainley,W.M., Walker,J.C., Nagao,R.T. and Key,J.L. (1988) *J. Biol. Chem.*, **263**, 10658–10666.

Akiyoshi,D.E., Klee,H., Amasino,R.M., Nester,E.W. and Gordon,M.P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5994–5998.

Alliotte,T., Tire,C., Engler,G., Peleman,J., Caplan,A., Van Montagu,M. and Inzé,D. (1989) *Plant Physiol.*, **89**, 743–752.

Badenoch-Jones,J., Summons,R.E., Entsch,B., Rolfe,B.G., Parker,C.W. and Letham,D.S. (1982) *Biomed. Mass Spectrom.*, **9**, 429–437.

Binns,A.N. and Thomashow,W.F. (1988) *Annu. Rev. Microbiol.*, **42**, 575–606.

Braune,W., Leman,A. and Taubert,H. (1983) *Pflanzenanatomisches Practicum.* Fischer-Verlag, Jena, Vol. I, p. 241.

Conner,T.W., Goekjian,V.H., LaFayette,P.R. and Key,J.L. (1990) *Plant Mol. Biol.*, **15**, 623–632.

Davies,P.J. (1987) *Plant Hormones and Their Role in Plant Growth and Development.* Martinus Nijhoff, Dordrecht.

Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.

Feldwisch,J., Zettl,R., Hesse,F., Schell,J. and Palme,K. (1991) *Proc. Natl. Acad. Sci. USA*, in press.

Gielen,J., DeBeuckeleer,M., Seurinck,J., Deboeck,F., DeGreve,H., Lemmers,M., Van Montagu,M. and Schell,J. (1984) *EMBO J.*, **3**, 835–846.

Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hesse,T., Feldwisch,J., Balshüsemann,D., Bauw,G., Puype,M., Vandekerckhove,J., Löbler,M., Klämbt,D., Schell,J. and Palme,K. (1989) *EMBO J.*, **8**, 2453–2461.

Inzé,D., Follin,A., Van Lijsebettens,H., Simeons,C., Genetello,C., Van Montagu,M. and Schell,J. (1984) *Mol. Gen. Genet.*, **184**, 265–274.

Jefferson,R.A. (1987) *Plant Mol. Biol. Rep.*, **5**, 387–405.

John,M., Schmidt,J., Wienke,U., Kondorosi,E., Kondorosi,A. and Schell,J. (1985) *EMBO J.*, **4**, 2425–2430.

Joos,H., Inzé,D., Caplan,A., Sormann,M., Van Montagu,M. and Schell,J. (1983) *Cell*, **32**, 1057–1067.

Kaper,J.M. and Veldstra,H. (1958) *Biochim. Biophys. Acta*, **30**, 401–420.

Koncz,C. and Schell,J. (1986) *Mol. Gen. Genet.*, **204**, 383–396.

Koncz,C., Martini,N., Mayerhofer,R., Koncz-Kalman,Zs., Körber,H., Redei,G.P. and Schell,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8467–8471.

Koncz,C., Mayerhofer,R., Koncz-Kalman,Zs., Nawrath,C., Reiss,B., Redei,G.P. and Schell,J. (1990) *EMBO J.*, **9**, 1337–1346.

Kramer,W., Drutsa,V., Jansen,H.W., Kramer,B., Pflugfelder,M. and Fritz,H. (1984) *Nucleic Acids Res.*, **12**, 9441–9456.

Linsmaier,E.M. and Skoog,F. (1965) *Physiol. Planta*, **18**, 100–127.

Maurel,C., Brevet,J., Barbier-Brygoo,H., Guern,J. and Tempé,J. (1990) *Mol. Gen. Genet.*, **223**, 57–64.

Mayerhofer,R., Koncz-Kalman,Zs., Nawrath,C., Bakkeren,G., Crameri,A, Angelis,K., Redei,G.P., Schell,J., Hohn,B. and Koncz,C. (1991) *EMBO J.*, **10**, 697–704.

McClure,B.A., Hagen,G., Brown,C.S., Gee,M.A. and Guilfoyle,T.J. (1989) *Plant Cell*, **1**, 229–239.

Nester,E.W., Gordon,M.P., Amasino,R.M. and Yanofsky,M.F. (1984) *Annu. Rev. Plant Phys.*, **35**, 387–413.

Nilsson,B., Abrahmsen,L. and Uhlen,M. (1985) *EMBO J.*, **4**, 1075–1080.

Palme,K., Hesse,T., Moore,I., Campos,N., Feldwisch,J., Garbers,C., Hesse,F. and Schell,J. (1991) *Mech. Dev.*, **33**, 97–106.

Phelps,R.H. and Sequeira,L. (1968) In Wightman,F. and Setterfield,G. (eds), *Biochemistry and Physiology of Plant Growth Substances.* Runge Press, Ottawa, pp. 197–211.

Rao,R.N. and Rogers,S.G. (1979) *Gene*, **3**, 11–13.

Reiss,B., Sprengel,R, Will,H. and Schaller,H. (1984) *Gene*, **30**, 211–218.

Rovenska,J., Kutacek,H., Opartny,Z. and Eder,J. (1982) In Wareing,P.F. (ed.), *Plant Growth Substances.* Academic Press, London, pp. 409–414.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandberg,G., Crozier,A. and Ernstsen,A. (1987) In Crozier,A. and Rivier,L. (eds), *Principles and Practice of Plant Hormone Analysis.* Academic Press, London, pp. 169–301.

Sandberg,G., Gardeström,P., Sitbon,F. and Olsson,O. (1990) *Planta*, **180**, 562–568.

Schell,J. (1986) In Gustafson,P.J., Stebbins,G.L and Ayala,F.J. (eds), *Genetics, Development and Evolution.* Plenum Press, New York, pp. 343–353.

Schell,J. and Kahl,G. (1984) *Molecular Biology of Plant Tumors.* Academic Press, New York.

Scott,T.K. (1984) *Hormonal Regulation of Plant Development II, Encyclopedia of Plant Physiology.* Vol. 10. Springer Verlag, Berlin.

Schröder,G., Waffenschmidt,S., Weiler,E.W. and Schröder,J. (1984) *Eur. J. Biochem.*, **138**, 387–391.

Sembder,G., Gross,D., Liebbisch,H.W. and Schneider,G. (1980) In Pirson,A. and Zimmermann,M.H. (eds), *Encyclopedia of Plant Physiology.* Springer-Verlag, Berlin. Vol. 9, pp. 281–444.

Sembder,G., Schneider,G. and Schreiber,K. (1988) *Methoden der Pflanzenhormonanalyse.* Fischer-Verlag, Jena.

Spanier,K., Schell,J. and Schreier,P.H. (1989) *Mol. Gen. Genet.*, **219**, 209–216.

Spena,A., Schmülling,T., Koncz,C. and Schell,J. (1987) *EMBO J.*, **6**, 3891–3899.

Stahl,E. (1967) *Dünnschichtchromatographie.* Springer-Verlag, New York.

Staiger,D., Kaulen,H. and Schell,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6390–6394.

Stark,M.J.R. (1987) *Gene*, **51**, 255–267.

Tabor,S. and Richardson,C.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4767–4771.

Tinland,B., Huss,B., Paulus,F., Bonnard,G. and Otten,L. (1989) *Mol. Gen. Genet.*, **219**, 217–224.

Trewavas,A. (1980) *Phytochemistry*, **19**, 1303–1308.

Trewavas,A. (1981) *Plant Cell Environ.*, **1**, 204–228.

Trinchant,J.C. and Rigaud,J. (1977) *C.R. Acad. Sci. Ser D*, **284**, 301–303.

Walden,R., Koncz,C. and Schell,J. (1990) *Methods Mol. Cell Biol.*, **1**, 175–194.

Wightman,F. (1973) *Biochem. Soc. Symp.*, **38**, 247–275.

Willmitzer,L., Dhaese,P., Schreier,P., Schmalenbach,W., Van Montagu,M. and Schell,J. (1983) *Cell*, **32**, 1045–1056.

Wing,D., Koncz,C. and Schell,J. (1989) *Mol. Gen. Genet.*, **219**, 9–16.

Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–109.

Zambryski,P. (1988) *Annu. Rev. Genet.*, **22**, 1–30.

Zambryski,P., Tempé,J. and Schell,J. (1989) *Cell*, **56**, 193–201.

Zimmer,W., Roeben,K. and Bothe,H. (1988) *Planta*, **176**, 333–342.

Received on July 2, 1991; revised on September 19, 1991