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Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants

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Abstract

The gene encoding the auxin-responsive GH3 mRNA (G. Hagen, A. Kleinschmidt, TJ. Guilfoyle, Planta 162: 147–153 (1984)) from soybean was cloned, and its sequence and transcription initiation site were determined. The promoter of the GH3 gene has been fused to the open reading frame of the *Escherichia coli uidA* gene which encodes β-glucuronidase (GUS). This fusion gene was introduced into tobacco via *Agrobacterium tumefaciens*-mediated transformation, and the expression of the gene was examined by fluorometric assay and histochemical staining of young R1 tobacco seedlings and mature plants. In transgenic tobacco plants that have not been exposed to exogenous auxin, expression of the fusion gene is largely restricted to roots of young green plants and developing floral organs, including ovules, developing seeds, and pollen, of mature plants. Application of exogenous auxin to tobacco seedlings or plant organs results in a greater than 50-fold increase in expression of GUS. Auxin-induced GUS expression is greatest in vascular tissue, but not restricted to this tissue. The auxin-deduced GUS expression was characterized for kinetics, auxin specificity and dose response.

Abbreviations: NAA – α-naphthaleneacetic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; 2,4-D – 2,4-dichlorophenoxyacetic acid; GUS – β-glucuronidase; NOS – nopaline synthase.

Introduction

The naturally occurring auxin, indole-3-acetic acid (IAA) and its synthetic derivatives elicit dramatic effects on plant growth and development when applied to plants or excised plant organs. In processes such as cell extension, auxin-induced growth effects are very rapid, occurring within minutes after auxin addition [5]. The mechanisms involved in auxin-induced growth and developmental changes are only poorly understood, and primary levels of control have been proposed to range from transcriptional to posttranslational [5, 8]. While auxins have been shown to regulate

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X60033.

a variety of different genes in plants [8, 12, 18], it is unclear what role, if any, the auxin-responsive gene products play in growth or developmental processes. Based on kinetics of induction as well as organ and tissue localization studies, some of the auxin-regulated gene products are thought to play roles in cell extension, while others are thought to play roles in cell division [8].

We have previously characterized an auxin-responsive mRNA which we have named GH3 [9]. The gene encoding this mRNA has been shown to be transcriptionally induced within 5 minutes after auxin is applied to plants or excised plant organs [10]. We used *in situ* hybridization to show that exogenous auxin induces the expression of GH3 mRNA primarily in vascular tissues in a wide variety of soybean organs [6]. Here, we report on the sequence of the soybean GH3 gene and on the auxin-induced expression of β-glucuronidase (GUS) in transgenic tobacco plants which contain a GH3 promoter fused to the open reading frame of the *Escherichia coli uidA* gene [16]. We conclude from these studies that 592 nucleotides of the GH3 promoter are sufficient to drive auxin-induced and tissue-specific GUS expression. We have characterized this auxin-induced expression of GUS for kinetics of induction, tissue specificity, auxin specificity and dose response in transgenic dark grown tobacco seedlings and green plants.

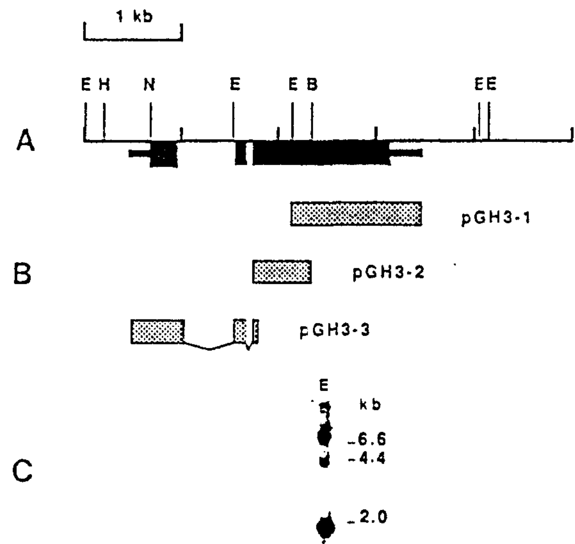

Fig. 1. Structure and organization of the GH3 gene. A. Restriction endonuclease map of the soybean genomic fragment containing the GH3 gene. The exons which encode the open reading frame are indicated by the solid boxes. Thick lines extending from the solid boxes indicate the 5′ and 3′ untranslated regions of the exons. Restriction endonuclease sites are indicated by B (*Bam* HI), E (*Eco* RI), H (*Hin*d III), and N (*Nco* I). B. Location of the sequence homologies of the pGH3 cDNAs in relation to the gene, as determined by sequence analysis. C. Southern blot hybridization of soybean genomic DNA probed with pGH3-1 cDNA. Soybean (cv. Wayne) genomic DNA (10 μg) was digested with *Eco* RI (E), fractionated in a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled pGH3-1 insert (1.2 kb). Size markers (lambda DNA digested with *Hin*d III) are indicated in kb at the right.

Materials and methods

Analysis of genomic DNA and isolation of genomic clones

Soybean genomic DNA was prepared from primary leaf nuclei, digested with *Eco* RI, electrophoresed in agarose gels, and transferred to nylon membranes as described by McClure *et al.* [19]. Using purified ^{32}P-labeled insert DNA from the pGH3-1 cDNA clone ([9]; see Fig. 1), gel blots of genomic DNA were hybridized, washed, and exposed to X-ray film [19].

A soybean (*Glycine max* cv. Wayne) genomic DNA library [11] was screened with pGH3-1 insert cDNA. Positive, purified phage DNA was isolated, and restriction fragments were subcloned into plasmid vectors pIBI24 and 25 (International Biotechnologies, New Haven, CT) or Bluescript SK^{+} and KS^{+} (Stratagene, LaJolla, CA).

cDNA cloning

To obtain a nearly complete cDNA sequence for GH3 mRNA, several overlapping cDNA clones were isolated from a cDNA library. The cDNA library was constructed using poly(A)$^{+}$ RNA

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                                                                      GAATTCACGAATAAAGAAAAATTAAAAG   28
TCTCAACAAATGTAGTAAGAGGGCAAAAATAGGCTGTAATAACTTGCAAAGTGTGCAGTGAAGTTTTCTTCGTACTACGTAGAAACTTCTCAGTTCTTTCTCA  131
CATTTCTGCCCACAGGGATTTGGATTTCGTGTATTGACGCAGTTATACCATCATTAATCTTATCCTTCAATTTTTATAAAATTAATAAAATAAATAAAAAATT  234
AATTAAGCTTCCGATCTTGACTGCCTGCTTGAATGCGTCGGCGGCGCCCATTAGTTTCTCATGCCAACACACCCTATAACGCCTAATTTTGCCCGAGTATTAC  337
TATATTGGGAGAACTTTTGCTGACGTGGCGACACATCTGGACCCACATGTCGGCCACCATGCACCATCCCTGGCCCTCGTGTCTCCTCAATAAGCTACACAAT  440
TTGAAACATACACGCAATCCTTTGTCTCAATAAGTTCCACTCAGGTACTGTTTTCTCCCGCAACCATGACGTAATTCTGTAAATCACATGTTTCATGCTCCCA  543

ATTATTTTCCGCTTCTATAAATACCTCTCCCATTTCGCAACTTTTCTCCATCCATACTCATCCACTTCTTGAACCGTGCCTTAACTAAACTAGAGCTAGAATT  646
AGAGTTAGCTACCTTGCCTAATTCACAAACGCGTCCCTCTACGGCTCTACCTATTAGCTATCTTTTTTGTGCTGTGATTGAAATTAATTTGTGATAGCTCACC  749
ATG GCC ATT GCT ACT TGT GAT CAT GAT AAG AAC GCA AAG GCT TTG CAG TTC ATT GAG GAT ATG ACC CAA AAC ACT GAA  827
 M   A   I   A   T   C   D   H   D   K   N   A   K   A   L   Q   F   I   E   D   M   T   Q   N   T   E   26
AGT GTC CAA GAG AGG GTC CTT GCT GAG ATT CTC AGC CAA AAC TCC CAG ACC GAA TAC CTG AAA CGG TTT GAG CTG AAT  905
 S   V   Q   E   R   V   L   A   E   I   L   S   Q   N   S   Q   T   E   Y   L   K   R   F   E   L   N   52
GGA GCC ACA GAC CGC GAC ACT TTC AAG TCA AAG GTT CCT GTC GTT TCC TAT GAT GAT TTG AAG CAT GAT ATC CAT CGC  983
 G   A   T   D   R   D   T   F   K   S   K   V   P   V   V   S   Y   D   D   L   K   H   D   I   H   R   78
ATT GCT AAT GGT GAC CGC TCT CCT ATT TTA TGC GCT CAC CCA ATC TCC GAG TTT CTC ACC AG gtacataaccttagcgcaca 1065
 I   A   N   G   D   R   S   P   I   L   C   A   H   P   I   S   E   F   L   T   S                        99
cacatatataatacatattatctctctctcttttttttttttttggcaaaagaactttctattattgaaaatatgagccaaggctggtacaagatgtaccag 1168
attagtccactagcattacaaagaaataaaaaaataacccaactttagaatgatggtgcgaaaaaaaaaccctccactgtacggtaaccaagaagagaaaa 1271
ggaaagaaattgtgagtttgaacctcctttactaataaaactaatattttgtccgtaaaaaaaaaaaagacaagatgcacgtctgcaatatatgcatatgtc 1374
attttctgcagttgtcaaactgcattgaacataaataaaacattgacctttattaaggtaaaattgtaattttaagtgtagtgtatatagtatcatatataat 1477
ttctttaatttgatatttttatatgaaaatatgaattctgtttgaatcagtgtgaattaattaagcagctaccgttaattaaattgcag T TCT GGA ACA 1576
                                                                                            S   G   T  102
TCT GCT GGG GAG AGA AAA TTG ATG CCA ACC ATT CGT CAA GAG ATG GAC CGT CGT CAA TTA ATT TTC AGC CTT CCC ATG 1654
 S   A   G   E   R   K   L   M   P   T   I   R   Q   E   M   D   R   R   Q   L   I   F   S   L   P   M  128
CCA GTG ATG AAT CA  gtatgtttccatgccttggttaattcccctttccctttacatatagatagtttggtaacttctgttttgtgtttatattcatg 1751
 P   V   M   N   Q                                                                                     133
tgcatgttgttacattacacag    A TAC GTG ACT GAT ATG GAC AAG GGT AAG GCT CTC ATC TTC CTG TTC ACC AAA GCC GAG 1831
                             Y   V   T   D   M   D   K   G   K   A   L   I   F   L   F   T   K   A   E  152
CAG AAA ACT CCG AGT GGC TTA GTG GCA CGT CCA GTG TCG GCT AGC ATG TAC AAG AGC GAC CAG TTC AAA AAC AGA CCC 1909
 Q   K   T   P   S   G   L   V   A   R   P   V   S   A   S   M   Y   K   S   D   Q   F   K   N   R   P  178
TAC GAC CCA TAC AAC GTG TAC ACA AGC CCA GAC GAA GCA ATC CTC TGC CCC GAT TCC TTC CAA AGC ATG TAC ACC CAA 1987
 Y   D   P   Y   N   V   Y   T   S   P   D   E   A   I   L   C   P   D   S   F   Q   S   M   Y   T   Q  204
ATG CTA TGC GGC CTC ATC ATG CGC CAT CAA GTC CTC AGA GTT GGT GCA AAT TTC GCC TCC GGC CTT CTC CGA TCC ATC 2065
 M   L   C   G   L   I   M   R   H   Q   V   L   R   V   G   A   N   F   A   S   G   L   L   R   S   I  230
CAC TTG CTC CAG CTC AAT TGG GCC CAA CTA TCC CAC GAC ATC TCC ACC GGA ACC CTA AAC CCC AAA ATC ACT GAC CCT 2143
 H   L   L   Q   L   N   W   A   Q   L   S   H   D   I   S   T   G   T   L   N   P   K   I   T   D   P  256
GCC ATC AAA CAA CGC ATG ACC CAA ATC CTC AAA CCC GAC CCA GAA CTT GCT GAA TTC ATT GTG AAA GAA TGC TCA GGA 2221
 A   I   K   Q   R   M   T   Q   I   L   K   P   D   P   E   L   A   E   F   I   V   K   E   C   S   G  282
GAA AAC TGG GAA CGC ATA ATC CCC AGA ATC TGG CCA AAC ACC AAG TAT GTA GAG GTG GTT GTG ACC GGT GCC ATG GCG 2299
 E   N   W   E   R   I   I   P   R   I   W   P   N   T   K   Y   V   E   V   V   V   T   G   A   M   A  308
CAG TAT GTT CCA ACT CTT GAT TAT TAC AGC GGA GGC CTA CCT CTC GCT TCC AAC ATA TAC GGA TCC TCC GAG TGC TTC 2377
 Q   Y   V   P   T   L   D   Y   Y   S   G   G   L   P   L   A   S   N   I   Y   G   S   S   E   C   F  334
TTT GGG ATT AAC CTC AAC CCA TTT TGC AAC CCC TCT GAC GTG TCA TAC ACC ATC ATG CCA AAC ATG GGT TAC TTC GAG 2455
 F   G   I   N   L   N   P   F   C   N   P   S   D   V   S   Y   T   I   M   P   N   M   G   Y   F   E  360
TTC TTG CCT CAG GAT CAC GAT GAT GCT TCT TCG TCT TCA GGT TCC AGT TTC ACT CTG TCG CGT TTA ATC GAC CTT GAT 2533
 F   L   P   Q   D   H   D   D   A   S   S   S   S   G   S   S   F   T   L   S   R   L   I   D   L   D  386
GAT GTT GAA CTC GGA AAA TCC TAC GAA ATT GTT GTG ACC ACG TAC TCC GGT TTA TGC CGT TAC CGT GTG GGC GAC ATT 2611
 D   V   E   L   G   K   S   Y   E   I   V   V   T   T   Y   S   G   L   C   R   Y   R   V   G   D   I  412
CTC CGA GTG ACG GGC TTC CAC AAC ACC GCC CCG CAG TTC AGC TTC GTG AGG AGA AAA AAC GTG TTG CTG AGC ATC GAC 2689
 L   R   V   T   G   F   H   N   T   A   P   Q   F   S   F   V   R   R   K   N   V   L   L   S   I   D  438
TCG GAC AAA ACG GAC GAA GCG GAG TTA CAA AAC GCC GTG GAG AAG GCC TCC GTG CTG TTG AAG GAG TTC AAG ACG AGC 2767
 S   D   K   T   D   E   A   E   L   Q   N   A   V   E   K   A   S   V   L   L   K   E   F   K   T   S  464
GTG GTG GAG TAC ACG AGC TTT GCG GAC ACG AAG TCG ATC CCG GGG CAT TAC GTG ATT TAC TGG GAG CTC TTG ATG AAG 2845
 V   V   E   Y   T   S   F   A   D   T   K   S   I   P   G   H   Y   V   I   Y   W   E   L   L   M   K  490
GAC TCT TCC AAC GCT CCA ACT ACT GAA GCG TTG GAG CAG TGT TGT TTA ACA ATG GAA GAA TCG CTG AAC GCC GTT TAT 2923
 D   S   S   N   A   P   T   T   E   A   L   E   Q   C   C   L   T   M   E   E   S   L   N   A   V   Y  516
CGA CAA GGT AGG GTT GCG GAC CAT TCT ATT GGA CCG TTA GAG ATT CGT GTG GTG AAG AAT GGA ACC TTT GAG GAG CTT 3001
 R   Q   G   R   V   A   D   H   S   I   G   P   L   E   I   R   V   V   K   N   G   T   F   E   E   L  542
ATG GAC TAC GCT ATC TCA CGT GGC GCG TCT ATT AGC CAG TAC AAG GTT CCA CGG TGC GTG ACC TTC ACG CCC ATA ACG 3079
 M   D   Y   A   I   S   R   G   A   S   I   S   Q   Y   K   V   P   R   C   V   T   F   T   P   I   T  568
GAG TTG CTC GAC TCT AGG GTT GAG TCC GTT CAT TTT AGC CCC TCT GAA CCA CAC TGG ACC CCG GAA CGA CGT CGT TGA 3157
 E   L   L   D   S   R   V   E   S   V   H   F   S   P   S   E   P   H   W   T   P   E   R   R   R       593
AGGGTAACTACACATACCTGCGTGAAGTCATGCGCTCAATAATTAAAATAAAGAGATTGAAAGGAATGTGCCTTTTCATTTTGGCCGTGCTTTTTAAAGTTTA 3260
TGTGTTATGTTTTAGATTTGTGTCTCTGAAAAATATGGTCAAAAGGAATTGTCCTAGACATCTTTGTCTATCCTATGTAGGAAACCATCATGTGCACACGTTT 3363
AATTAGTCGATCTATTTATAATGTTAATACATTATAATTTTAATATCCTTCACGTGCATGTAACTTTCATGGGGCCAAAAGTCTGAGAAACGGTTGTCTTTTC 3466
TCTTCTTTTTCGTGTAAAGTTAATTACCCGTATATAACATGAATTTGAGTTGAATTAATAAAGTCGTTATAATGTTTCAGATGTGAAACTCAAACTCCAAACA 3569
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Fig. 2. Nucleotide and deduced amino acid sequence of the GH3 gene. Nucleotides are numbered from the 5′ *Eco* RI site (see Fig. 1). The transcription start site, as determined by primer extension analysis, is indicated by an arrow. The deduced amino acid sequence is shown in single-letter code below the DNA sequence. Intron sequence is shown in lower case. Underlined sequences in the 5′ and 3′ flanking regions of the open reading frame are discussed in the text (see Results and Discussion).

from 2,4-D-treated soybean primary leaves [10]. Based on the sequence of the 5′ end of pGH3-1 insert (See Figs. 1 and 2), an 18 nucleotide oligomer was synthesized, annealed to the mRNA, and used to prime the synthesis of double-stranded cDNA (cDNA Synthesis System Plus, Amersham, Arlington Heights, IL). The cDNA was inserted into λgt10 [15] by the addition of *Eco* RI adaptors. The inserts of two purified, primer-extended cDNA clones of pGH3-1, designated pGH3-2 and pGH3-3 (see Fig. 1), were subcloned into Bluescript vectors.

DNA sequencing

The cDNA insert of pGH3-1, originally cloned into pBR322 [9], was recloned into M13, mp18 and mp19. The DNA sequence of both strands of the cDNA and genomic clones was obtained by the dideoxy chain termination procedure [22], using overlapping deletions [4] and synthetic primers.

Primer extension analysis

Analysis of the 5′ end of the GH3 transcript was performed using the primer extension procedure described by Arndt *et al.* [3]. A 15 nucleotide oligomer was used to prime the synthesis of a 68 nucleotide ^{32}P-labeled probe ending at the *Nco* I site in the translation start site of GH3 (see Fig. 2). The probe was annealed to poly(A)$^+$ RNA (4.8 μg) isolated from 2,4-D-treated soybean primary leaves. The primer-extended products were analyzed in a sequencing gel.

GH3 promoter constructs and plant transformation

A cloned 1.5 kb *Eco* RI genomic fragment containing the 5′ untranslated and 5′ upstream region of the GH3 gene was restricted with *Sal* I and *Nco* I, and a 749 bp fragment was cloned into the GUS vector, pEBGUS. The pEBGUS vector was constructed by inserting the *Bam* HI-*Eco* RI fragment of the GUS open reading frame and nopaline synthase (NOS) 3′ untranslated region from pAGUS1 [24] into pUC19 (C. Brown, unpublished results). The GH3 promoter-pEB-GUS construct contains 592 bp of the GH3 5′ upstream region from an *Eco* RI site to the transcription start site and the GH3 5′ leader sequence fused at its translation start site (*Nco* I) to the ATG of the GUS open reading frame. The GH3 promoter-GUS open reading frame-NOS 3′ untranslated region insert was isolated from a *Sal* I/partial *Eco* RI digest and ligated into pMON505 [14], a disarmed Ti plasmid that was digested with *Xho* I and *Eco* RI. This pMON construct was mobilized into the binary vector, *Agrobacterium* pTi37-SE, using the triparental mating method, and transformed into *Nicotiana tabacum* cv. Xanthi by the leaf disk method as described by Horsch *et al.* [13] and Rogers *et al.* [21]. Transformed shoots were rooted, transferred to soil, and grown in growth chambers.

Seedling and plant organ section incubations

R1 tobacco seeds were imbibed and germinated on moist sand in Magenta boxes for 24 h at room temperature on a laboratory bench. The boxes were then transferred to a dark chamber, and the seedlings were grown for four to six days. The etiolated tobacco seedlings were removed from the sand, washed briefly in distilled water, and transferred to 50 ml flasks containing 20 ml of MS Basal Salt Mixture (0.086 g; Sigma, St. Louis, MO), 10 mM Pipes buffer (pH 6.0), 50 μg/ml chloramphenicol, with or without added auxins (as indicated in the figures and figure legends). The flasks were incubated in the dark on a gyratory shaker for the times indicated.

For older green plants, organs were sectioned with a razor blade, and the sections were incubated as described above. Petioles and stems were excised as 1 mm cross sections and leaves were excised and cut into 0.5 cm $\times$ 1 cm planar sections prior to incubation with or without auxin.

GUS assays in transgenic tobacco plants

The GUS activity in transgenic tobacco seedlings was assayed as described by Jefferson [16]. Extracts were prepared from 1–3 g of seedlings which had been frozen in liquid nitrogen and kept at −80 °C until processing. Seedlings were homogenized in 2 to 6 ml of extraction buffer [16] using a Brinkman Polytron, and the homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA). The cleared extract was transferred to 1.5 ml microfuge tubes and centrifuged at 14000 for 15 min in a refrigerated Eppendorf microcentrifuge. Aliquots from the supernatant were incubated at 37 °C for 10 and 30 min in extraction buffer containing 1 mM 4-methylumbelliferyl glucuronide. GUS activity was determined fluorometrically with a Hoeffer Fluorometer. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce, Rockford, IL).

Histochemical staining was carried out in 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 1 mM X-gluc (Diagnostic Chemicals Limited, Charlottetown, P.E.I., Canada). Seedlings or sectioned organs were incubated at 25 or 37 °C for 12–24 h, and then cleared in 70% ethanol.

Results

Nucleotide sequence, gene structure, and transcription start site analysis of the soybean GH3 gene

The insert DNA fragment of the cDNA clone, pGH3-1 (referred to as pGH3 previously [9]), was used to screen a soybean genomic DNA library. One of several different purified genomic clones was characterized further. Restriction digest and hybridization analyses identified a 4 kb *Hin*d III fragment with homology to pGH3-1. Sequence analysis of both strands of the cDNA insert and the genomic DNA fragment localized the pGH3-1 sequence within the 4 kb *Hin*d III genomic DNA fragment (Figs. 1A, 1B, and 2). The structure of the GH3 gene was further elucidated by the isolation and sequence analysis of two additional cDNA clones, pGH3-2 and pGH3-3 (Fig. 1B) and by mapping the transcriptoin start site. Primer extension analysis revealed a single major transcription start site (Fig. 3). An untranslated leader sequence of 157 nucleotides precedes the first initiation codon. The coding information of the gene is contained within 3 exons (Fig. 2) and translates to a polypeptide of 593 amino acids. The cDNA clone, pGH3-1, contained a 3′ untranslated region of 245 nucleotides following the translation stop codon. While this cDNA clone did not contain a poly(A) tail, a putative polyadenylation signal is located 366 nucleotides down stream of the stop codon (underlined in Fig. 2). The pGH3-1 cDNA clone has been previously shown to hybridize to a mRNA of 2.4 kb, and the size of the mRNA (minus the poly(A) tail) based on sequence analysis is 2.3 kb (assuming the polyadenylation site to be 366 nucleotides from the translation stop codon).

Several lines of evidence suggest that the GH3 gene is part of a small multigene family in soy-

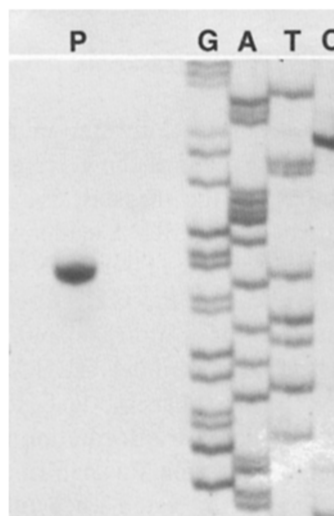

Fig. 3. Primer extension analysis of the 5′ end of GH3 mRNA. A 68 nucleotide ^{32}P-labeled probe complementary to the 5′ end of the open reading frame was annealed to 4.8 μg of poly(A)$^+$ RNA and extended with reverse transcriptase. The primer extended products (P) were analyzed in a 7% denaturing acrylamide gel in parallel with dideoxy sequencing reactions (G, A, T, C) that were generated using the same template and 15 nucleotide oligomer which was used to synthesize the 68 nucleotide probe.

bean. Hybridization of the pGH3-1 insert DNA to an *Eco* RI digest of soybean genomic DNA (Fig. 1C) revealed a major band of 1.9 kb, which corresponds to the *Eco* RI fragment in the genomic clone (Fig. 1A) containing the GH3-1 sequence. Several *Eco* RI restriction fragments greater than 1.9 kb were also observed to hybridize with pGH3-1. These other hybridizing fragments probably correspond to other GH3-like genes. That additional genes related to GH3 are in the soybean genome is supported by our isolation of two other genomic clones that hybridize with pGH3-1, but contain different restriction maps than that shown in Fig. 1. We also have evidence that more than one GH3-like mRNA is expressed in soybean, since we have isolated and sequenced a cDNA clone with 80% identity to pGH3-1 (data not shown).

We have searched sequence data banks to determine if the GH3 open reading frame has similarity to any other published amino acid or nucleotide sequence. No sequence similarity was identified in this search.

GH3 promoter analysis in transgenic tobacco plants

Based on the sequence information described above, a 749 bp fragment of the GH3 gene, containing 592 bp 5′ to the transcription initiation site and 157 bp of the 5′ mRNA leader, was fused to the translation start site of the GUS open reading frame. At its 3′ end, the GUS open reading frame was fused to the 3′ untranslated region of NOS [24]. This GH3 promoter-GUS fusion was used to transform tobacco, leaf disks via *Agrobacterium*-mediated tranformation (see Materials and methods). Primary transformants were selected on kanamycin, tested for expression of GUS with or without auxin treatments, and grown to maturity (Ro plants). Histochemical staining of plant organs from the primary transformants revealed that developing ovules and developing seeds, along with vascular tissues in the flower receptacle, showed the strongest expression of GUS in the absence of any auxin treatment (data not shown). Other organs showed little, if any, expression of GUS, unless exogenous auxin was applied to the Ro plants. When GUS activity was observed in organs not treated with auxin, the histochemical staining was localized primarily to young roots and trichomes on leaves, stems, petioles, and flower organs.

R1 tobacco seeds from seven independent primary transformants were germinated in light and then grown in the dark for 5 days, and the 5-day old etiolated seedlings were transferred to incubation medium containing or not containing 10 μM NAA. After a 24-hour incubation, the seedlings were frozen in liquid nitrogen, homogenized in buffer [16], and assayed for GUS activity using a 4-methylumbelliferyl glucuronide as substrate. The GUS activity for the individual transformants is shown in Fig. 4. Most R1 seed-

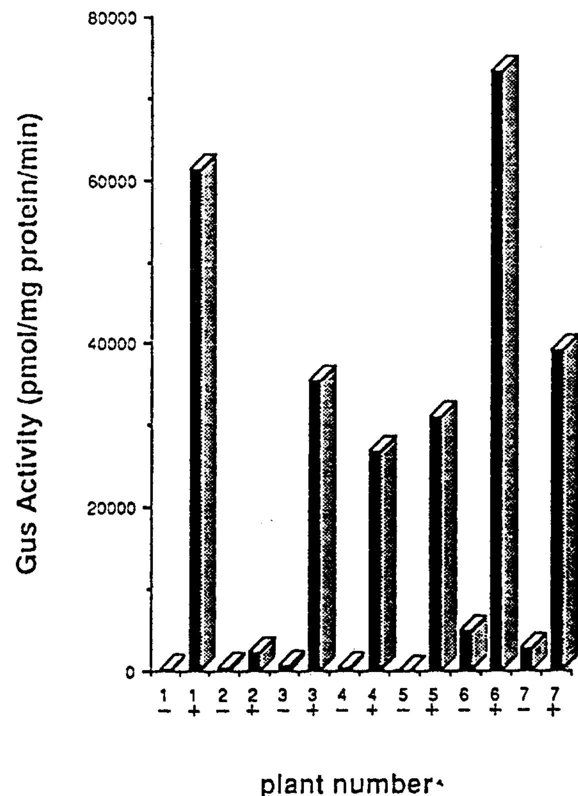

Fig. 4. GUS activity in transgenic tobacco plants. R1 seedlings from seven Ro independent transformants were incubated in media without (–) or with 10^{-5} M NAA (+) for 24 hours. GUS activity was determined by a fluorometric assay with tissue homogenates.

lings showed little, if any, GUS expression before or after incubation in a medium lacking auxin. R1 seedlings from transformants 6 and 7 did, however, show some GUS activity in the absence of auxin treatment. The increase in GUS activity following auxin treatment ranged from 5-fold for plant 3 to 200-fold for plants 1 and 5. Most of the primary transformants appear to have a single chromosomal site for the introduced gene based on 3:1 segregation of the R1 progeny for GUS expression (data not shown). Southern analysis of the R1 seedlings also indicates that one or possibly two transgenes are carried by the transformed plants expressing auxin-inducible GUS activity (data not shown).

For further analysis of the auxin response in transgenic seedlings, we chose to use transgenic plants 3 and 4 which show about a 50-fold induction in response to a 24-hour treatment with 10 μM NAA. R1 progeny from other primary transformants were analyzed in less detail than plants 3 and 4, but when comparisons were made with other transgenic tobacco plants, responses (e.g. dose response, kinetics, auxin-specificity) were observed similar to those which we report below. When young etiolated R1 seedlings, containing the soybean GH3 promoter fused to the GUS gene, are stained for GUS activity, little if any expression from the GH3 promoter can be detected in any part of the seedlings. Application of 10 μM NAA results in high levels of GUS expression throughout various organs of the transgenic seedlings (Fig. 5A). With this auxin treatment, the greatest amount of GUS expression is detected in the root/shoot transition zone and in the hypocotyl. Expression of GUS is also detected in the tips of cotyledons and the base of the cotyledons where these organs are fused to the apical portion of the hypocotyl (Fig. 5B). Within the root and hypocotyl tissues, GUS is most strongly expressed in the vascular tissues, but substantial expression of GUS is also observed in the cortex and epidermis.

In transgenic seedlings, auxin causes a relatively linear increase in GUS activity over a 24-hour period of incubation, but little further increase in GUS activity occurs over an additional 12-hour period (Fig. 6). A small increase (i.e., 10–20% above 0 time controls) in GUS activity can be detected as early as 2 h after hormone application, and a greater than 3-fold increase is observed within 4 h after auxin addition. By 24 h after auxin addition, the GUS activity is approximately 70-fold higher than the activity observed for seedlings before auxin addition or for seedlings incubated for 24 h in the absence of auxin. The kinetics for the auxin-induced increase in GUS activity in tobacco seedlings are very similar to the kinetics for GH3 protein accumulation in excised 2,4-D-treated organs (i.e., hypocotyl, root, and plumule) of soybean seedlings using anti-GH3 antibody [26]. Histochemical staining of the tobacco seedlings shows that the tissue and organ distribution of GUS activity is not altered over the 24-hour incubation period (data not shown), suggesting that temporal, sequential, or transient patterns in tissue or organ-specific gene expression do not occur during the auxin treatment.

A variety of different auxins induce expression from the GH3 promoter in transgenic tobacco seedlings (Fig. 7). At the auxin concentration of 50 μM, the most effective inducers are the synthetic auxins 2,4-D and NAA, while the naturally occurring auxin, IAA, and its synthetic analogue, IBA, are less effective inducers of GUS activity. A survey of other types of plant hormones (i.e., cytokinins, gibberellins, and abscisic acid) or inactive auxin analogues indicates that the only inducers are active auxins (data not shown).

We have analyzed the dose-response of seedlings and mature organ sections for a variety of auxins, including 2,4-D, NAA, and IAA. Figure 8 shows the dose-response for R1 transgenic tobacco seedlings treated with NAA. Induction of GUS activity is detected with as little as 10^{-8} M NAA (i.e. about 3-fold more GUS activity than observed for seedlings incubated in the absence of auxin), and expression from the GH3 promoter increases over several orders of log increase in NAA, up to 10^{-4} M. Induction with NAA decreases dramatically at concentrations greater than 10^{-4} M. The large decrease in GUS activity at 10^{-3} M NAA is not nearly as dramatic

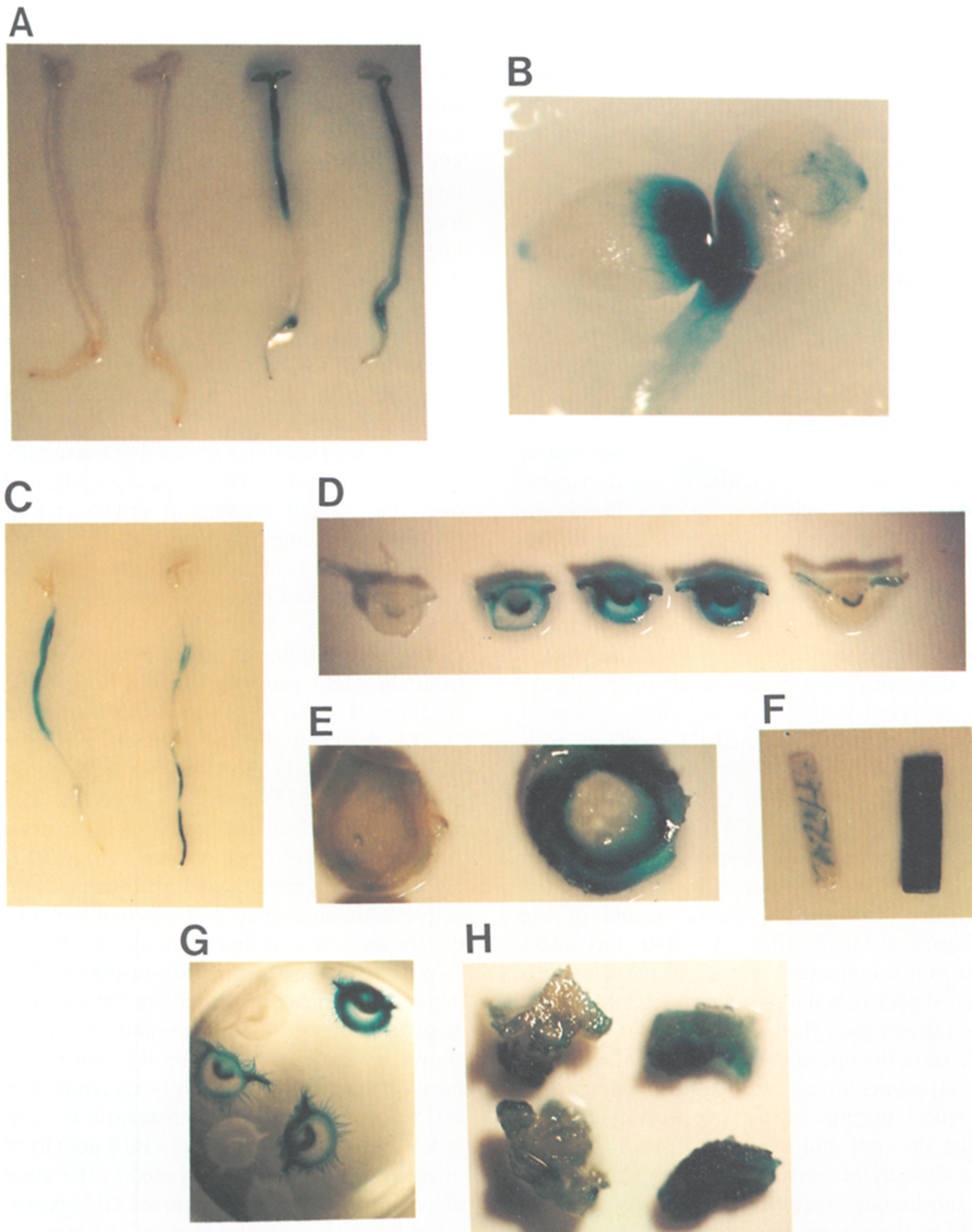

Fig. 5. Histochemical staining for GUS activity in transgenic R1 tobacco seedlings and organs. A. Seedlings incubated without (two seedlings on left) or with 10^{-5} M NAA (two seedlings on right). B. Cotyledons of a seedling incubated with 10^{-4} M NAA. C. Seedling incubated with 10^{-5} M IAA (left) or 10^{-7} M IAA (right). D. Petiole cross sections incubated with 0, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} M NAA (from left to right). E. Stem cross-sections incubated without (left) or with 10^{-5} M NAA (right). F. Leaf sections incubated without (left) or with 10^{-5} M NAA (right). G. Petiole sections incubated without or with 10^{-6} M NAA. H. Tissue cultures incubated without (left) or with 10^{-5} M NAA (right). All incubations were for 24 hours at 25 °C.

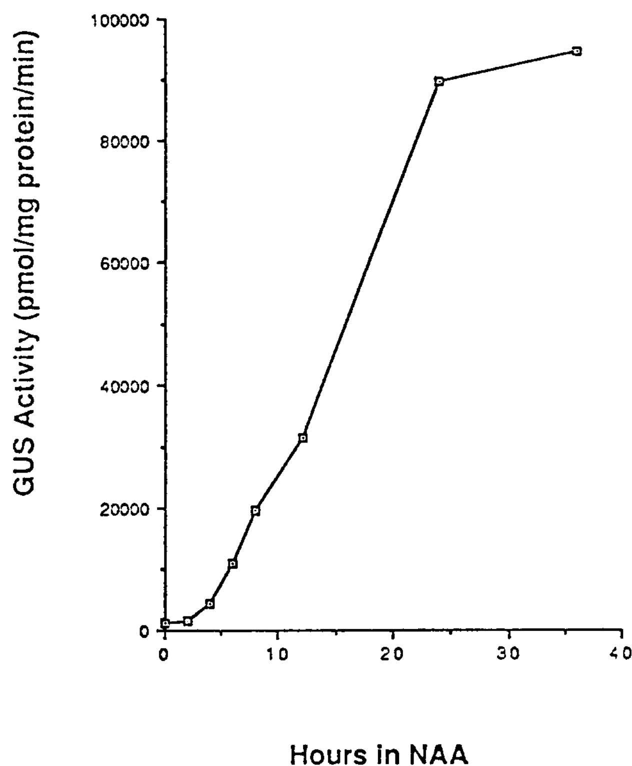

Fig. 6. Kinetics for appearance of GUS activity after applying 10^{-5} M NAA to R1 transgenic tobacco seedlings. GUS activity was determined by a fluorometric assay with tissue homogenates.

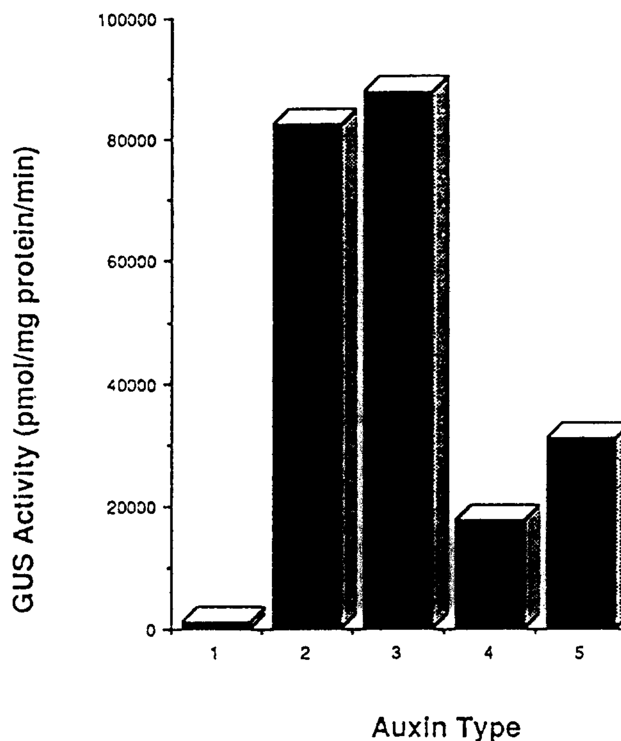

Fig. 7. GUS activity induced with different auxins. Lane 1: no auxin; lane 2: NAA; lane 3: 2,4-D; lane 4: IAA; lane 5: IBA. Auxin concentration was 50 μM. Incubations were with R1 transgenic tobacco seedlings for 24 h at 25 °C. GUS activity was determined by a fluorometric assay with tissue homogenates.

when 2,4-D is used in place of NAA, and 10^{-3} M IAA is observed to be even more effective than 10^{-4} M IAA when this naturally occurring auxin is substituted for NAA (data not shown).

We have observed that the pattern of expression in different organs of the seedlings varies with the dose of auxin. Figure 5C shows that at low concentrations (i.e., 10^{-6} to 10^{-7} M) of IAA, GUS expression is detected primarily in the root, including the root tip. At higher auxin concentrations (i.e., 10^{-4} to 10^{-5} M), expression of GUS becomes much greater in the hypocotyl and decreases in the root. We have also observed that the dose-response with etiolated seedlings differs from that observed with petiole or stem sections which have been excised from green plants and incubated with different concentrations of auxins. With these excised sections, a maximal response is obtained with 10^{-6} to 10^{-5} m NAA or 2,4-D and concentrations in excess of 10^{-5} M are strongly inhibitory (Fig. 5D).

Examination of a variety of different organ sections of green plants indicates that petioles, stems, and leaves express the GH3 promoter in response to applied auxin. In petioles (Fig. 5D) and stems (Fig. 5E), the greatest auxin-induced expression occurs in the vascular tissue, but the epidermis and cortex also express the auxin-induced gene. We also generally observe some expression in the pith of stems although this is not evident in Fig. 5E. In leaves, the GH3 promoter is expressed in vascular as well as surrounding tissues (Fig. 5F). A small amount of GUS expression can be detected in the vascular tissues of uninduced organs, and this is especially evident in Fig. 5F. Low levels of GUS expression are sometimes detected in the tips of trichomes found on

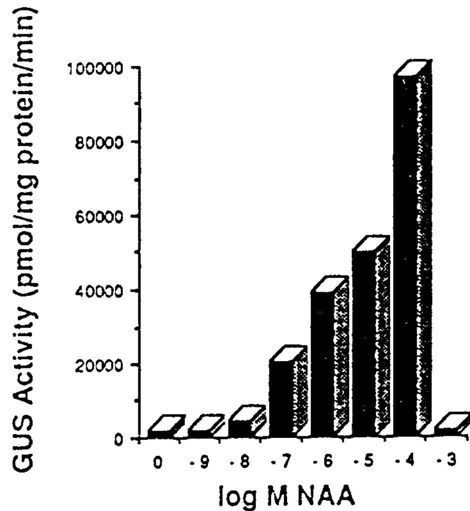

Fig. 8. Dose-response for auxin-induced GUS activity in transgenic R1 tobacco seedlings. Seedlings were incubated for 24 hours at 25 °C in the NAA concentration indicated. GUS activity was determined by a fluorometric assay with tissue homogenates.

stems, petioles, and leaves, and application of auxin causes a large increase throughout these surface cells (Fig. 5G). In addition to differentiated seedlings and green plant organs, we have observed that tissue culture cells also express the GH3 promoter in an auxin-dependent manner (Fig. 5H).

In young green transgenic tobacco plants, we have observed GUS expression in some, but not all roots. While this expression is generally restricted to the root tips, we occasionally observe expression extending up the roots within the vascular tissue (data not shown). In mature tobacco plants, we have observed the expression of GUS in developing flower parts. Some of the floral organs transiently express the GH3 promoter in the absence of applied auxin. This is especially evident in ovules and developing seeds. Immature ovules and seeds express GUS throughout, but as the seeds mature, this expression becomes restricted to the funiculus which connects the developing seed to the placenta (Fig. 9A–E). At later stages of seed maturation, the expression of the GH3 promoter is not observed. Fully mature seeds show no GUS activity. However, upon germination, we routinely observe some GUS expression in the seed coat where the radicle breaks through (data not shown). Strong GUS expression is also observed in late stages of pollen development and in mature pollen grains (Fig. 9F). Finally, the vascular tissues of the receptacle and the basal regions of other floral organs (e.g., sepals, petals) show a relatively high level of GUS expression (data not shown). We have not attempted to further induce expression of the GH3 promoter in floral organs by applying exogenous auxins.

Discussion

We have shown that a 592 bp promoter region from the auxin-inducible soybean GH3 gene functions as an auxin-inducible element in transgenic tobacco seedlings and plants. We are currently carrying out a detailed deletion analysis of the GH3 promoter to better define the auxin-inducible element(s). We have noted a number of specific sequence elements within this promoter which might participate in the regulation of its expression (underlines in the 5′ upstream region of Fig. 2). A TATA box consisting of **TATAAATA** is located −34 to −27 from the transcription start site. The GH3 promoter contains three potential TGA1 [17, 25] binding sites within 450 bp from the start site of transcription. These sites are related to the AP1 or CREB sequences identified in other eukaryotes [20]. Two of these sites contain the sequences **TGACGTGG** and **TGACGTAA** and are identical to one or the other TGA1 binding sites characterized in other plant genes [17, 25]. The third, most 5′, site contains the sequence **TGACGCAG**. Since TGA1 is thought to function in root-specific expression, one or more of these sites within the GH3 promoter may be involved with the auxin-inducible gene expression observed in roots and root-shoot transition zones. A second notable sequence element in the GH3 promoter is **GTCGGCGGCG--CCCATTaGT** which is strikingly similar to the sequence GTCGGCGGCGggtCCCATTtGT found in the auxin-inducible *Agrobacterium*

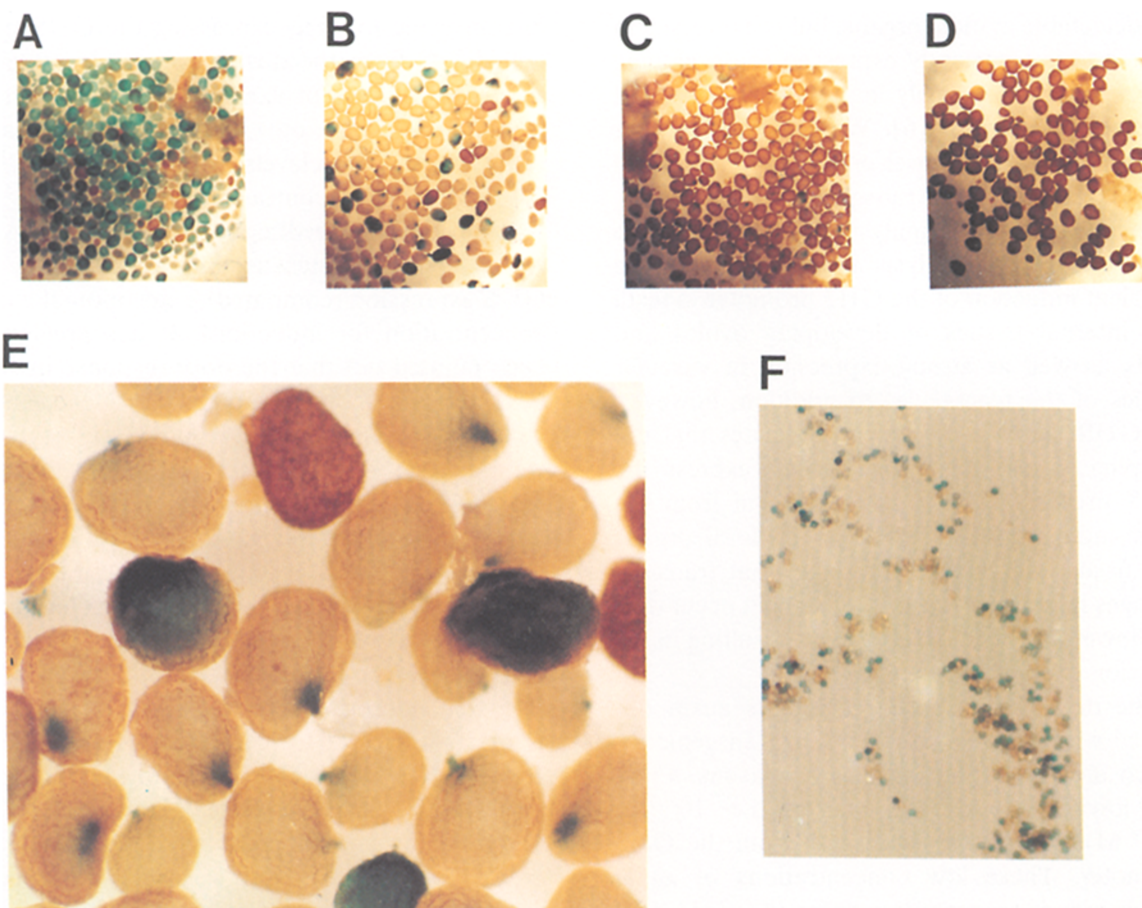

Fig. 9. Histochemical staining for GUS activity in developing seeds and pollen of R1 transgenic tobacco. Developing seeds progressing in maturity from A to C and mature seeds D are shown. Increased magnification of B is shown in E. Mature pollen is shown in F.

tumefaciens gene 5 promoter [7] and the sequence GTaGGCGGgtgcgtCCCATTatT found in the auxin-inducible *A. rhizogenes rolb/c* promoter [23]. The major difference among these sequences is the spacing between the GC-rich and the CCCATT elements. Sequence similarities to other auxin-responsive genes have also been examined, and, while striking similarities are not found, certain sequence elements are worth noting. Specifically, two overlapping sequence elements, cCACCATGC and GCACCATcC in GH3, are similar to an element in the B′ box of Aux 28 [1], **GCACCATGC**, and the 5′ region of the NDE element of SAURs X15 and 6B [19], aCACCATat. In the broader context, these CACCAT motif sequences fit the consensus element found by An *et al.* [2] to be present in a variety of auxin-inducible genes.

We have shown that the GH3 promoter is specifically induced by active auxins in a dose-dependent and tissue-specific manner in a variety of different organs of transgenic tobacco plants. The tissue-specific pattern of expression induced by exogenous auxins, with strongest expression in the vascular tissues and weaker expression in cortex, pith and epidermis, is identical to the tissue-specific expression pattern of the GH3 gene in soybean observed by *in situ* hybridizations [6]. *In situ* hybridization analysis of GH3 gene expression in soybean revealed that in the absence of exogenous auxin application, GH3 mRNA is

not detectable in most organs, but is expressed in roots and is transiently expressed in developing flower organs, especially in vascular tissues and in developing ovules [6]. We observe a similar pattern of root and flower organ expression from the GH3 promoter in transgenic tobacco plants. Both the *in situ* RNA analysis in soybean and the GUS expression analysis in tobacco reveal a transient induction of the GH3 promoter in testa and internal tissues of developing ovules and seeds as well as strong expression in vascular tissues of the receptacle. In addition, however, the GUS analysis in tobacco indicates that developing and mature pollen grains express the GUS protein. The strong expression from the auxin-inducible GH3 promoter in floral organs and tissues raises that possibility that transient changes in auxin concentrations might occur during flower and seed development, resulting in expression of the GH3 gene.

The dose-responses to exogenous auxin observed with the GH3 promoter in transgenic tobacco are interesting for several reasons. First, very low levels of exogenous auxin (i.e., 10^{-7} to 10^{-8} M) can induce expression from the GH3 promoter. These low concentrations of auxin which induce transcription from the GH3 promoter in tobacco are 10–100-fold less than the concentrations of auxin previously observed to induce transcription of the GH3 gene [10] or accumulation of the GH3 protein [26] in excised organ sections of soybean seedlings. The apparent greater sensitivity of tobacco to auxin is likely due to the greater sensitivity of the GUS assay system used in tobacco compared to the nuclear run-on and immunological assays previously used in soybean. Second, the dose-response differs in tobacco seedlings compared to excised stems and petioles in that seedlings require a 10–100-fold higher auxin concentration than excised stems and petioles for maximal expression from the GH3 promoter. Third, in etiolated tobacco seedlings, induction of expression from the GH3 promoter occurs at lower concentrations in the root than in the hypocotyl or cotyledons, and at higher auxin concentrations, expression is stronger in hypocotyls than roots. Fourth, the dose response in transgenic tobacco expressing the GUS protein differs from the dose response reported in soybean seedlings or excised hypocotyls, where maximal response of GH3 transcription and steady-state mRNA levels are observed at 10^{-3} m 2,4-D [9, 10]. In contrast to soybean, 10^{-3} M NAA in tobacco seedlings and 10^{-4} M NAA in tobacco petiole or stem sections are inhibitory for GUS expression (compared to the optimal auxin concentration for induction). It has previously been pointed out that the dose response in soybean appears to be nonsaturable with exogenous auxin [8]. While the dose responses in soybean were conducted with 2,4-D [10, 26], in tobacco seedlings, the results shown in Fig. 8 are with NAA as the auxin. We have observed, however, that 10^{-3} M 2,4-D is only slightly less effective than 10^{-4} M 2,4-D and that 10^{-3} M IAA is more effective than 10^{-4} M IAA in tobacco seedlings, both of which contrast markedly from NAA (Fig. 8). Concentrations in excess of 10^{-3} M 2,4-D or IAA were observed to be as inhibitory as 10^{-3} M NAA in tobacco seedlings. Thus, our results with transgenic tobacco indicate that different auxins and different plant systems (e.g., excised mature organs versus whole young seedlings, roots versus hypocotyls of young seedlings) give different dose-responses. Our results further suggest that analysis of tissue- and organ-specific gene expression induced by auxin does not provide a complete picture if only a single hormone or a single concentration of a hormone is evaluated.

We are currently attempting to define the minimal element in the GH3 promoter that allows induction by exogenous auxin. In addition, we are interested in determining whether the putative TGA1 sites or other sequence elements regulate organ and tissue-specific expression of the GH3 promoter and whether these elements can be separated from the auxin-inducible element(s). Finally, we would like to determine whether the auxin-responsive element(s) in the GH3 promoter are identical, similar, or different from auxin-inducible elements in other auxin-responsive genes.

Acknowledgements

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References

1. Ainley WM, Walker JC, Nagao RT, Key JL: Sequence and characterization of two auxin-regulated genes from soybean. J Biol Chem 263: 10658–10666 (1988).
2. An G, Costa MA, Ha S-B: Nopaline synthase promoter is wound inducible and auxin inducible. Plant Cell 2: 225–233 (1990).
3. Arndt KT, Styles CA, Fink GR: A suppressor of a HIS4 transcriptional defect encodes a protein with homology to the catalytic subunit of protein phosphatases. Cell 56: 527–537 (1989).
4. Dale RMK, McClure BA, Houchins JP: A rapid single-stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: Application to sequencing corn mitochondrial 18S rDNA. Plasmid 13: 31–40 (1985).
5. Evans ML: The action of auxin on plant cell elongation. CRC Crit Rev Plant Sci 2: 317–365 (1985).
6. Gee MA, Hagen G, Guilfoyle TJ: Tissue-specific and organ-specific expression of soybean auxin-responsive transcripts GH3 and SAURs. Plant Cell 3, in press (1991).
7. Gielen J, De Beuckeleer M, Seurinck J, Deboeck F, De Greve H, Lemmers M, Van Montagu M, Schell J: The complete nucleotide sequence of the TL-DNA of the *Agrobacterium tumefaciens* plasmid pTiAch5. EMBO J 3: 835–846 (1984).
8. Guilfoyle TJ: Auxin-regulated gene expression in higher plants. CRC Crit Rev Plant Sci 4: 247–276 (1986).
9. Hagen G, Kleinschmidt A, Guilfoyle T: Auxin regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta 162: 147–153 (1984).
10. Hagen G, Guilfoyle T: Rapid induction of selective transcription by auxins. Mol Cell Biol 5: 1197–1203 (1985).
11. Hagen G, Uhrhammer N, Guilfoyle TJ: Regulation of expression of an auxin-induced soybean sequence by cadmium. J Biol Chem 263: 6442–6226 (1988).
12. Hagen G: Molecular approaches to understanding auxin action. New Biol 1: 19–23 (1989).
13. Horsch RB, Fry JE, Hoffmann NL, Wallroth M, Eichholtz D, Rogers SG, Fraley RT: A simple and general method for transferring genes into plants. Science 227: 1229–1231 (1985).
14. Horsch RB, Klee HJ: Rapid assay of foreign gene expression in leaf discs transformed by *Agrobacterium tumefaciens*: Role of T-DNA borders in the transfer process. Proc Natl Acad Sci USA 83: 4428–4432 (1986).
15. Huynh TV, Young RA, Davis RW: Constructing and screening cDNA libraries in lambda gt10. In: Glover DM (ed) DNA Cloning Techniques: A Practical Approach, vol 1, pp. 49–78. IRL Press, London (1985).
16. Jefferson RA: Assay for chimeric genes in plants: the GUS fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
17. Katagiri F, Lam E, Chua N-H: Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature 340: 225–233 (1989).
18. Key JL: Modulation of gene expression by auxin. BioEssays 11: 52–58 (1989).
19. McClure BA, Hagen G, Brown CS, Gee MA, Guilfoyle TJ: Transcription, organization and sequence of an auxin-regulated gene cluster in soybean. Plant Cell 1: 229–239 (1989).
20. Mitchell PJ, Tjian R: Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245: 371–378 (1989).
21. Rogers SG, Klee HJ, Horsch RB, Fraley RT: Improved vectors for plant tranformation: Expression cassette vectors and new selectable markers. Meth Enzymol 153: 253–277 (1987).
22. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain termination inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
23. Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D: Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J Biol Chem 261: 108–121 (1986).
24. Skuzeski JM, Nichols LM, Gesteland RF: Analysis of leaky viral translation termination codons *in vivo* by transient expression of improved β-glucuronidase vectors. Plant Mol Biol 15: 65–79 (1990).
25. Tabata T, Takase H, Takayama S, Mikami K, Nakatsuka A, Kawata T, Nakayama T, Iwabuchi M. A protein that binds to a cis-acting element of wheat histone genes has a leucine zipper motif. Science 245: 965–967 (1989).
26. Wright RM, Hagen G, Guilfoyle T: An auxin-induced polypeptide in dicotyledonous plants. Plant Mol Biol 9: 625–643 (1987).