The Elicitor-Inducible Alfalfa Isoflavone Reductase Promoter Confers Different Patterns of Developmental Expression in Homologous and Heterologous Transgenic Plants

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In legumes, the synthesis of infection- and elicitor-inducible antimicrobial phytoalexins occurs via the isoflavonoid branch of the phenylpropanoid pathway. To study transcriptional regulation of isoflavonoid pathway–specific genes, we have isolated the gene encoding isoflavone reductase (IFR), which is the enzyme that catalyzes the penultimate step in the synthesis of the phytoalexin medicarpin in alfalfa. Chimeric gene fusions were constructed between 765- and 436-bp promoter fragments of the *IFR* gene and the β-glucuronidase reporter gene and transferred to alfalfa and tobacco by Agrobacterium-mediated transformation. Both promoter fragments conferred elicitor-mediated expression in cell suspension cultures derived from transgenic plants of both species and fungal infection–mediated expression in leaves of transgenic alfalfa. Developmental expression directed by both promoter fragments in transgenic alfalfa was observed only in the root meristem, cortex, and nodules, which is consistent with the accumulation of endogenous IFR transcripts. However, in transgenic tobacco, expression from the 765-bp promoter was observed in vegetative tissues (root meristem and cortex, inner vascular tissue of stems and petioles, leaf tips, and stem peripheries adjacent to petioles) and in reproductive tissues (stigma, placenta, base of the ovary, receptacle, seed, tapetal layer, and pollen grains), whereas the 436-bp promoter was expressed only in fruits, seed, and pollen. These data indicate that infection/elicitor inducibility of the *IFR* promoter in both species and developmental expression in alfalfa are determined by sequences downstream of position −436, whereas sequences between −436 and −765 confer a complex pattern of strong ectopic developmental expression in the heterologous species that lacks the isoflavonoid pathway.

INTRODUCTION

Phytoalexins are low molecular weight antimicrobial compounds synthesized by plants in response to attempted infection by fungal pathogens, exposure to elicitor macromolecules, or other biotic and abiotic stresses (Dixon et al., 1983). Isoflavonoid phytoalexins are characteristic of the Leguminosae and are found very rarely in other plant families (Dewick, 1988). In tobacco and other solanaceous species, terpenoid compounds derived from the isoprenoid pathway are the major phytoalexins; isoflavonoids do not appear to be produced in the Solanaceae (Kuć, 1982).

The pterocarpan medicarpin is the major phytoalexin in the forage legume alfalfa. It is synthesized from L-phenylalanine via the central phenylpropanoid pathway and isoflavonoid branch pathway as illustrated in Figure 1. The entire biosynthetic pathway to medicarpin is induced de novo in whole plants and cell cultures when subjected to the appropriate infection or elicitation conditions (Higgins, 1972; Paiva et al., 1991, 1994a).

Genes encoding early enzymes in the phytoalexin biosynthetic pathway, such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), have been cloned from several plant species, and their developmental and environmental expression patterns have been studied using promoter–reporter gene fusions (Liang et al., 1989; Ohl et al., 1990; Stermer et al., 1990; Fritze et al., 1991). The complex expression patterns of these genes are consistent with the involvement of the corresponding enzymes in the synthesis of a wide range of functionally distinct phenylpropanoid-derived secondary products. The promoters of several PAL and CHS genes share common regulatory cis elements, consistent with the coordinated transcriptional activation of these genes at the onset of the isoflavonoid phytoalexin response.

In contrast, little is known about the molecular mechanisms underlying the induction of the isoflavonoid branch pathway. Enzymes specific to isoflavonoid phytoalexin biosynthesis have recently been characterized from several members of the Leguminosae. In alfalfa, isoflavone reductase (IFR; EC 1.3.1.45) catalyzes the NADPH-dependent reduction of 2'-hydroxyformononetin to vestitone, which is the penultimate step in the synthesis of medicarpin (Figure 1). Subsequent reduction and
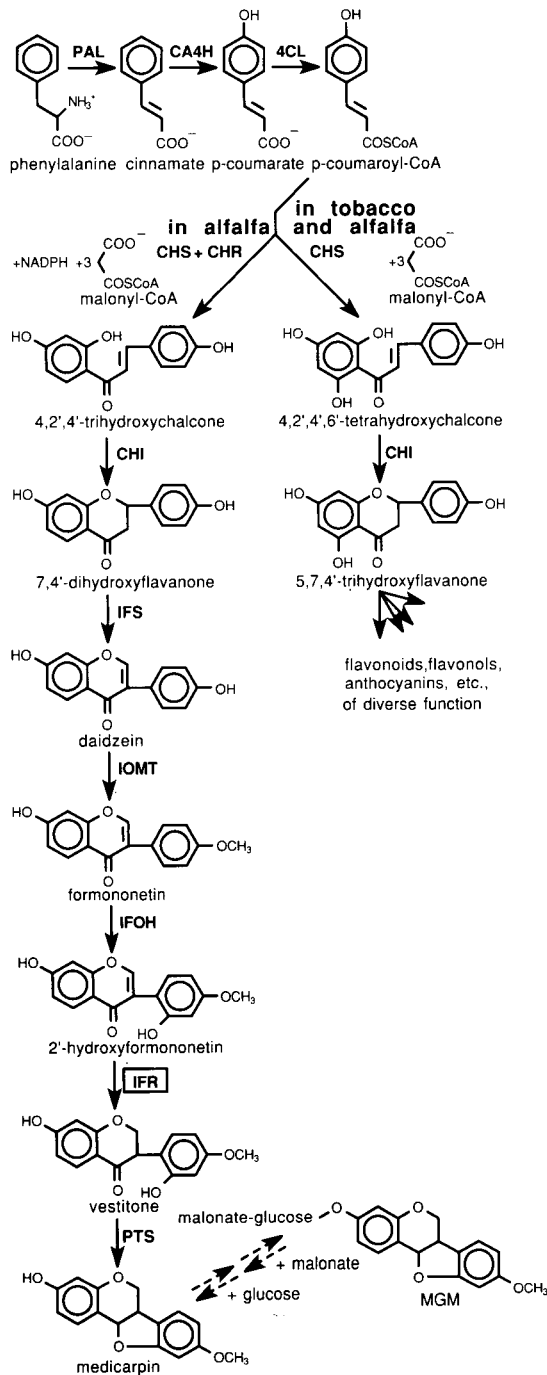
Figure 1. Biosynthesis of Medicarpin in Alfalfa in Relation to General Flavonoid Biosynthesis.

The isoflavonoid phytoalexin medicarpin is synthesized in legumes by a specific branch of phenylpropanoid metabolism. Non-legumes, such as tobacco, do not possess chalcone reductase, isoflavone synthase, or other enzymes of isoflavonoid metabolism. The enzymes represented are PAL, L-phenylalanine ammonia-lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; CHS,

chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; IOMT, isoflavone O-methyl transferase; IFOH, isoflavone 2'-hydroxylase; IFR, isoflavone reductase; PTS, pterocarpan synthase. The medicarpin conjugate MGM (medicarpin-3-O-glucoside-6″-O-malonate) accumulates constitutively in alfalfa roots and young cell cultures at low levels.

ring closure convert vestitone to medicarpin. IFR has been purified and characterized from several legumes, and IFR cDNA clones have been obtained from alfalfa (Paiva et al., 1991), chick-pea (Tiemann et al., 1991), and pea (Paiva et al., 1994b). In unstressed alfalfa plants, transcripts from the single alfalfa *IFR* gene are detected mainly in roots and root nodules, consistent with the accumulation of a medicarpin conjugate, medicarpin-3-O-glucoside-6″-O-malonate, only in these organs (Paiva et al., 1991). IFR transcripts are, however, strongly induced in infected leaves or elicited cell cultures at the onset of medicarpin accumulation (Paiva et al., 1994a).

Because IFR is specific for the synthesis of defense-related isoflavonoid compounds, the *IFR* gene promoter may respond to a more limited set of signals than do *PAL* and *CHS* gene promoters. Understanding how the *IFR* gene is activated will provide insights into the mechanisms underlying the coordinated transcriptional activation of a complex secondary biosynthetic pathway and the evolution of the isoflavonoid branch of flavonoid metabolism as a defense mechanism. Furthermore, the *IFR* promoter may be useful in biotechnology for driving expression of antimicrobial proteins or biosynthetic enzymes in transgenic plants. In this study, we report the isolation of the alfalfa *IFR* gene and its developmental and elicitor/infection–induced expression in transgenic alfalfa and in tobacco, a species that lacks the isoflavonoid pathway. A 436-bp region of the *IFR* promoter confers the predicted elicitor/infection inducibility plus the correct root- and nodule-specific developmental expression in transgenic alfalfa. Surprisingly, a 329-bp region upstream of this elicitor-response region directs similar patterns of expression in roots of transgenic tobacco but strong ectopic expression in shoots and floral organs.

RESULTS

Transcriptional Activation of *IFR* in Response to Elicitation

To ensure that the increase in IFR transcripts observed in alfalfa cell cultures after elicitation (Paiva et al., 1991) is a result of increased transcriptional activation, steady state transcript levels were determined by RNA gel blot analysis, and changes in IFR transcription were confirmed by run-on analysis using isolated nuclei. The data in Figure 2 indicate that the massive increase in IFR transcripts observed 3 hr after exposure of cells to elicitor from yeast cell walls is preceded by a striking

A   **B**
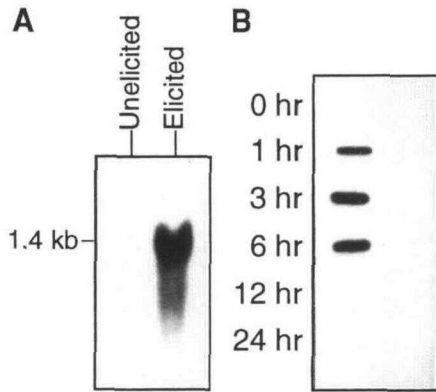
Figure 2. Transcriptional Activation of *IFR*.

(A) RNA gel blot from unelicited and elicited alfalfa cell suspension cultures probed with a labeled internal HindIII fragment (containing coding sequences for IFR) of pAO1-1. RNA was isolated from cell cultures that were exposed to yeast elicitor for 3 hr. The approximate size of the IFR mRNA band is indicated at the left.

(B) Run-on transcription from nuclei isolated from alfalfa cell suspension cultures treated with yeast elicitor for the times indicated. Slot blot analysis was performed with immobilized cDNA specific to IFR (pIFRalf1).

increase in the transcription rate from an undetectable initial level. Approximately equal transcription rates were observed at 3 and 6 hr after elicitation, but transcription had ceased by 12 hr after elicitation. Essentially similar transcription kinetics, with a maximum at 3 hr, were observed for PAL (data not shown).

Isolation and Characterization of the Alfalfa *IFR* Gene

A probe corresponding to the majority of the coding region of the alfalfa IFR cDNA (pIFRalf1; Paiva et al., 1991) was used to isolate clones from an alfalfa genomic library. Restriction mapping and DNA gel blot hybridization with four positive clones revealed strongly hybridizing 2.4-kb EcoRI and 1.4-kb HindIII fragments in each case, which was similar to the pattern observed in DNA gel blot analysis of total alfalfa genomic DNA (Paiva et al., 1991). The hybridizing 2.4-kb EcoRI fragment from one phage clone was subcloned into pBluescript II SK− and designated pAO1-1. Sequence analysis revealed that this clone contained the entire coding region of *IFR* but only 528 bp 5′ to the open reading frame. A 5′ end–specific probe (XbaI-HindIII fragment of pIFRalf1) was used to identify an overlapping 4-kb HindIII fragment that was also subcloned into pBluescript II SK−. A 2-kb PstI fragment was removed from the 5′ end to produce pAO4-1. The complete nucleotide sequence of pAO1-1 (EcoRI to EcoRI) and the additional 5′ flanking promoter sequence from pAO4-1 (329 bp; SpeI to EcoRI) are shown in Figure 3. The positions of four introns were deduced by comparison with the cDNA sequence of pIFRalf1. The splice points conform to the "GT-AG" rule for donor and acceptor sites (Breathnach and Chambon, 1981). The nucleotide sequences

of the deduced coding regions of pAO1-1 and the cDNA pIFRalf1 were 98.1% identical and the corresponding protein sequences were 99.1% identical, with only one functionally different amino acid substitution.

The start of transcription was mapped by primer extension analysis (Figure 4) to 92 nucleotides from the start of translation. Transcripts from elicited (Figure 4, lane 4) and unelicited (lane 5) alfalfa cell suspension cultures together with transcripts from alfalfa roots (lane 3) gave the same major primer extension product. In elicited cells, a minor reverse transcription product two nucleotides longer was also observed, probably indicating a second start of transcription from the next available site. A "TATA box" is located 32 nucleotides upstream from the transcription start site, and two possible "CAAT box" elements are located at positions −151 (GTCAATTT) and −113 (CAAT).

Alfalfa *IFR* Promoter–β-Glucuronidase Fusions in Transgenic Plants

Promoter sequences of the alfalfa *IFR* gene were fused to the β-glucuronidase (*GUS*) gene in the binary vector pBI101.1 (Jefferson et al., 1987) as outlined in Figure 5. Transgenic plants transformed with the construct pAlf-ifrL–GUS, containing the longer (765-bp) promoter fragment, are referred to as "ifrL–GUS" plants. Similarly, transgenic plants transformed with pAlf-ifrS–GUS, containing the shorter promoter fragment (a 329-bp deletion to the EcoRI site within the longer promoter), are referred to as "ifrS–GUS" plants. Both constructs are transcriptional fusions to the *GUS* gene in which 80 nucleotides of the 92-nucleotide untranslated leader sequence of the *IFR* transcript was maintained intact. These two constructs, along with pBI121 (cauliflower mosaic virus [CaMV] 35S promoter–*GUS* for constitutive expression controls) and pBI101.1 (as a control for *GUS* with no promoter) were introduced into tobacco and alfalfa plants by Agrobacterium-mediated plant transformation. These are referred to as "35S–GUS" and "promoterless GUS" plants, respectively. DNA gel blot analysis was used to determine whether regenerated plants were transformants; both a *GUS*-specific probe (BamHI-SacI fragment of pBI101.1) and an *IFR* promoter–specific probe (the 845-bp SpeI fragment from pAO4-1) were used. Border analysis indicated a range of one to six transgene copies in tobacco and one to four copies in alfalfa. Restriction enzyme digestion of genomic DNA to release internal fragments of the integrated binary constructs, followed by DNA gel blot analysis, revealed hybridizing bands of the expected sizes, indicating that no rearrangements of the constructs had occurred.

Of eight ifrL–GUS independent tobacco transformants with the same qualitative GUS staining pattern, four plants (T8, T9, T11, and T14) showed higher levels of staining with 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) and were used in all studies. All seven independent transgenic tobacco plants obtained by transformation with the short promoter construct showed the same staining pattern as described below. Additional tobacco transformants generated in a later series of

-765 <u>ACTAGT</u>TTTGTAAGAATTTTTTGAAACTTGTGTAATCCAATATTAAAAAA
      SpeI

-715 TGTAAAAAAAAATGTTATCTTTTATACAAAACTTACCTTTTATGTTTCTT

-665 TAACTAATGCCTTAAAGATACCGATTAACATCCAATAATTAAATACCACC

-615 TAACATCAACAATTACAAAGAAATAACAACCTATTGAAACTCATTGCAAA

-565 CGCTCTAACTTGCAAACTTTCTTTTGAGAAAGTATTTTTTATTTAACTTT

-515 CTAGGTGTTGAAGAACAATTTATGTTGAGTGAATATTAAACACATTTTTT

-465 TATAATAGTTGAATCTATCAAATGAAGAC<u>GAATT</u>CAACATGCAGGTTGGG
                                   EcoRI

-415 TTGTGTCATTGTTAAAAAGTTGTGAAGTAAAGGTTTCAAGTTGAATATTT

-365 AAAAAATCCTTAAAAAAGTTATATGTATATATCATGTTAATAATAATAAT

-315 TAGTATAAATCGGTGTATTCTTTTGTTCTCTTTGCTAAGATATATTCTTG

-265 CTTCCGGCCAAGTTTTCAGCAGAATTGTTTGATAAGTAGAGTTTTTTTAT

-215 ATATATTTTAACTGACTACTAATATGTTTTATACGGAGTTAATTAAGTAG

-165 ACTTAAGAGAAGGCGTCAATTTTGACCAACAGGGCTGCTTCTATTTCAAC

-115 AACAATGAATATTAAATTTGGTCACTAAAACACACGAGAGTAGTAGATG

-65   GATTGAAGTTGGTGGCAATCCAAGTTTGTCC<u>TATAAAT</u>ATCAAACAAAGT
                                     ↳

-15   ATAGCTATTCATCACACACTCACTACTACTTTGGTAACGTATTCAAAACA

36   AGAAAAAACAGACAAAAACATAAACACACTTGTTTTTTT<u>ACTAGT</u>TATTT
                                              SpeI

86   TTTTCCAATGGCAACTGAAAACAAAATCCTGATCCTAGGACCAACAGGAG
            M   A   T   E   N   K   I   L   I   L   G   P   T   G   A

136   CTATTGGAAGACACATAGTTTGGGCAAGTATTAAAGCAGGAAATCCAACA
        I   G   R   H   I   V   W   A   S   I   K   A   G   N   P   T

186   TATGCTTTGGTTAGAAAAACACCTGGCAATGTTAACAAGCCAAAGCTTAT
        Y   A   L   V   R   K   T   P   G   N   V   N   K   P   K   L   I

236   TACAGCTGCTAATCCTGAAACCAAGGAAGAGCTTATTGATAATTACCAAT
        T   A   A   N   P   E   T   K   E   E   L   I   D   N   Y   Q   S

286   CTTTAGGAGTTATTCTACTTGAAgtaagtgatttcaatatgtgaaataat
        L   G   V   I   L   L   E

336   tttatattctatatatttattaaattgacctaatcaatatgtctttgact

386   ctgcagGGTGATATAAATGATCATGAAACTCTTGTTAAGGCAATCAAGCA
            G   D   I   N   D   H   E   T   L   V   K   A   I   K   Q

436   AGTTGACATTGTGATCTGTGCTGCTGGTAGACTACTAATTGAAGATCAGG
        V   D   I   V   I   C   A   A   G   R   L   L   I   E   D   Q   V

486   TCAAGATTATTAAAGCAATTAAAGAAGCTGGAAACGTTAAGgtgaacaaa
        K   I   I   K   A   I   K   E   A   G   N   V   K

536   tttgtcactacaccagtaaataagtccaaataagtcaattcatatagagt

586   cttagttagtaataactctttgatggttagatttgtactcgttatattga

636   atagtggtactaaatttcttgtgtcgacagAAATTTTTCCCATCTGAATT
                                        K   F   F   P   S   E   F

686   TGGGCTAGACGTGGACCGTCATGAGGCCGTTGAGCCAGTTAGACAAGTTT
        G   L   D   V   D   R   H   E   A   V   E   P   V   R   Q   V   F

736   TTGAAGAAAAAGCAAGTATCCGAAGAGTAATTGAAGCCGAAGGAGTTCCT
        E   E   K   A   S   I   R   R   V   I   E   A   E   G   V   P

786   TACACTTACCTTTGTTGCCACGCCTTTACCGGTTACTTCTTACGTAACTT
        Y   T   Y   L   C   C   H   A   F   T   G   Y   F   L   R   N   L

836   GGCTCAACTCGACACAACTGATCCTCCTCGGGACAAAGTTGTCATTCTTG
        A   Q   L   D   T   T   D   P   P   R   D   K   V   V   I   L   G

886   GAGATGGAAATGTGAAAGgtaacagacttagtcacagaacaattcaacaa
        D   G   N   V   K   G

936   actagtattgaacaaaagacacacaattcagttgtttcaataattatacc

986   ttactcatttcagGAGCATATGTAACTGAGGCTGATGTGGGAACTTTTAC
                        A   Y   V   T   E   A   D   V   G   T   F   T

1036 CATTAGAGCAGCAAATGATCCCAACACATTGAACAAAGCTGTCCATATTA
        I   R   A   A   N   D   P   N   T   L   N   K   A   V   H   I   R

1086 GACTCCCCGAAAATTATTTGACCCAAAATGAGGTCATTGCCCTTTGGGAG
        L   P   E   N   Y   L   T   Q   N   E   V   I   A   L   W   E

1136 AAAAAGATTGGGAAGACTCTTGAGAAAACTTATGTTTCAGAGGAACAAGT
        K   K   I   G   K   T   L   E   K   T   Y   V   S   E   E   Q   V

1186 TCTCAAGGATATTCAAGgtcagtaaaataaacgctttataaatattgtta
        L   K   D   I   Q   E

1236 agaattttttacaccggtaatcaatcatagttgataaatcgttaaaaatat

1286 ttgatttttaattatatctattttaatgaccgcacaaatatctgacggtgt

1336 atcaaaattaatctcttagtgttaaattatgagtgacatgtatgtcattt

1386 tacagcaattttgtaaaattaatcatgaaatatgttacttgctatgcagA

1436 ATCTTCATTCCCTCATAACTATTTGTTGGCATTGTACCATTCACAACAAA
        S   S   F   P   H   N   Y   L   L   A   L   Y   H   S   Q   Q   I

1486 TAAAAGGAGATGCAGTGTATGAGATTGATCCAGCCAAAGATATTGAAGCT
        K   G   D   A   V   Y   E   I   D   P   A   K   D   I   E   A

1536 TCTGAAGCCTATCCAGATGTGACATACACCACTGCTGATGAATATTTGAA
        S   E   A   Y   P   D   V   T   Y   T   T   A   D   E   Y   L   N

1586 TCAATTTGTCTAACGAATGCTAAGGAAATGTTCAATAAGACAATGAATTT
        Q   F   V   -

1636 AAAAAAAAAAAAAGTTTCACATCTGTGTATGTTTCTTGTGTTTGTTTAGTT

1686 TTGTTCTCAGTAATCCCTCCCAATTGATGT<u>AATAATT</u>TACAAA<u>AATAATA</u>

1736 <u>AAT</u>ATTATATTCTGTTCCACTGTTTGCACATCTTTGTCTCTTTGTTCAAT
        ↑

1786 ATTTTACATTGTGGCTTCTCATTTTATGCGTCACTGTGAAGGGCCGACTC
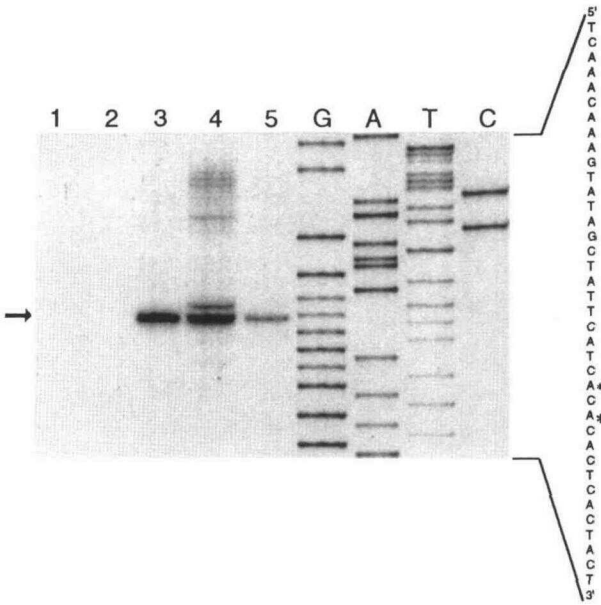
1836 CAAAAATAATTAAACGCACGCCCAAAATGGACTGAAAAATTCACTAATTA

1886 GACAAGTAGAAATATAATAAGAACTGAAATAATGACGAAAAAAAAAATAAG

1936 AACTAAAAAAAATAGAAATATTAGG<u>AATTC</u> 1965
                              EcoRI

Figure 3. Nucleotide Sequence of the Alfalfa *IFR* Gene.

The sequence of the promoter, 5' and 3' untranslated regions, and exons are indicated in uppercase letters (EMBL accession number U17436). Introns are indicated in lowercase letters. The deduced amino acid sequence is shown below the exon sequences in single-letter code. The transcription initiation site, as determined by primer extension, is marked with a bent arrow (position +1). Possible TATA box (−34 to −28) and polyadenylation signals (+1716 to +1721 and +1729 to +1737) are underlined, and the polyadenylation site is marked with an arrow (+1753). The restriction sites (EcoRI and SpeI) that were used to generate the promoter-*GUS* fusions are shown along with the two EcoRI sites that span the 2.4-kb EcoRI fragment of pAO1-1. The sequence upstream of the 5' EcoRI site was derived from pAO4-1.

The sequence at right of Figure 4 reads (5' to 3'):

```
5'
T
C
A
A
A
C
A
A
A
G
T
A
T
A
G
C
T
A
T
T
C
A
T
C
A *
C
A *
C
A
C
T
C
A
C
T
A
C
T
3'
```

Figure 4. Determination of the Transcription Start Site for the *IFR* Gene.

Primer extension analysis was done on total RNA isolated from alfalfa cell suspension cultures that were unelicited (lane 5) or elicited with yeast elicitor (lane 4) or from alfalfa roots (lane 3). The arrow shows the location of the major extension product. Total RNA from yeast was used as a control (lane 2), and lane 1 contains only the labeled primer. The same primer that was used for the primer extension reaction was used to generate a sequence ladder with pAO1-1 (lanes G, A, T, and C). The sequence of the region that was mapped is shown at right. The larger asterisk marks the primary transcription start site. The smaller asterisk indicates a possible minor transcription start site (see lane 4).

transformations were used to confirm the staining patterns observed in the roots of ifrL–GUS and ifrS–GUS plants. In the case of alfalfa, all transgenic plants obtained by transformation with the long or short promoter constructs showed the same staining pattern. Three independent transformants that showed strong staining were selected to represent each promoter construct (A3, A4, and A7 for the long promoter and A8, A9, and A11 for the short promoter).

Developmental Expression of *IFR* Promoter–*GUS* Fusions in Transgenic Alfalfa

Transgenic alfalfa plants were screened for observable GUS histochemical staining in roots. Both the long and short promoter fragments conferred identical *GUS* expression (Figures 6A and 6B). A zone of intense staining was seen in the region immediately behind the root tip, proximal to the quiescent center; the root cap and distal root meristematic region showed no GUS staining. Initiating lateral roots also showed high levels of GUS staining (Figure 6A). Transverse sections through

roots from both ifrL–GUS and ifrS–GUS plants revealed that GUS activity was localized exclusively to the inner cortex (Figure 6B). This pattern of *GUS* expression is clearly different from that of the CaMV 35S promoter in alfalfa roots, which is characterized by strongest GUS activity in the central vasculature (Figure 6C) and strong staining throughout the cortex and root tip, including the root cap. Transverse sections through stems of transgenic ifrL–GUS (Figure 6D) and ifrS–GUS (data not shown) alfalfa plants showed no detectable GUS activity. In comparison, transgenic plants obtained by transformation with pBI121 (35S promoter–*GUS*) showed staining in the vascular bundle, particularly in the secondary phloem (Figure 6E). There was no histochemically detectable GUS activity in any shoot parts (stems, leaves, or petioles), flower parts including pollen, or seed from ifrL–GUS or ifrS–GUS alfalfa plants, whereas pBI121-transformed plants showed intense staining in all tissues analyzed. Transformed plants containing pBI101.1 (promoterless *GUS*) did not show any detectable GUS activity.

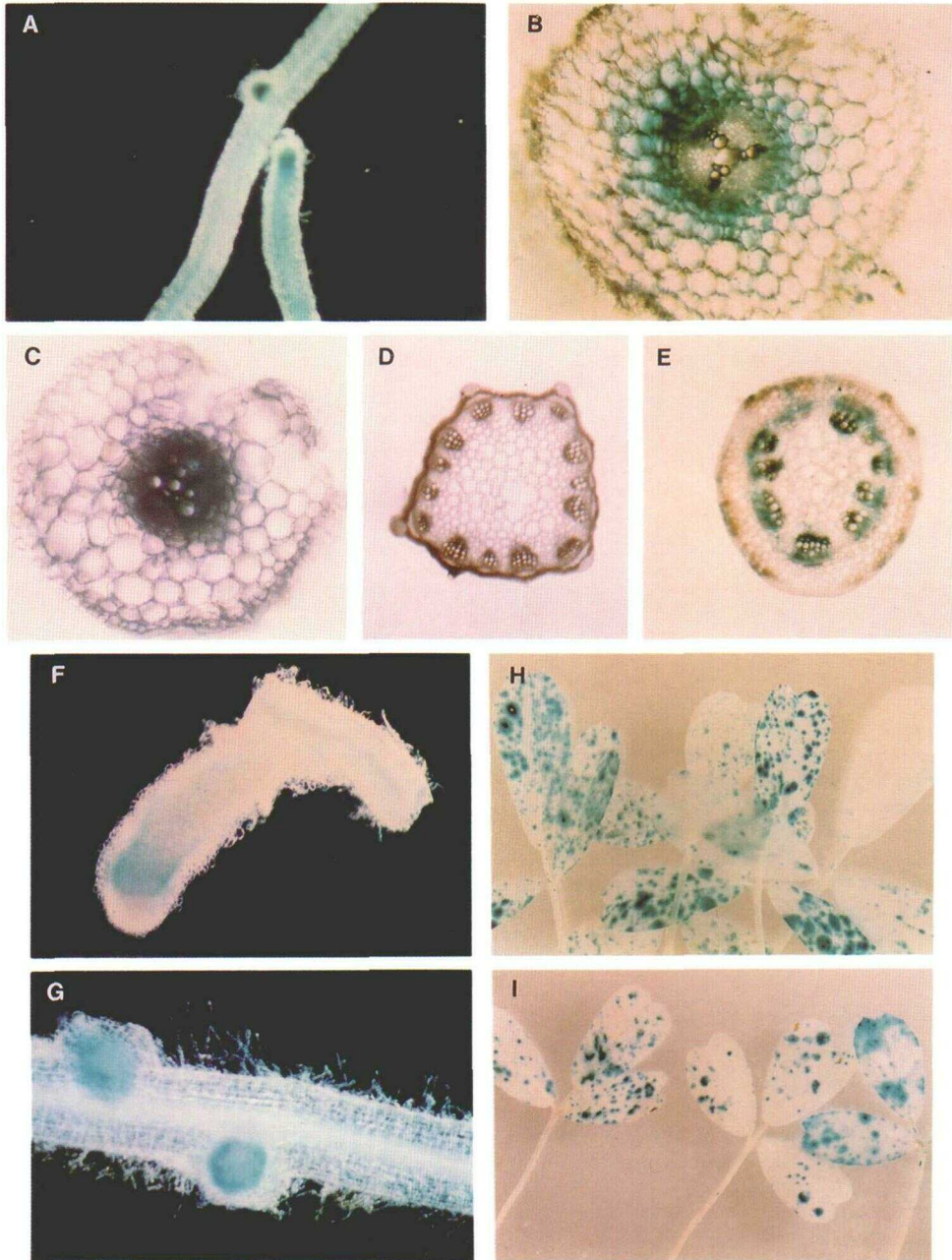## Expression of *IFR* Promoter-*GUS* Fusions in Alfalfa Root Nodules

The effective *Rhizobium meliloti* 102F51 readily formed nodules on rooted cuttings of transgenic alfalfa. Histochemical analysis revealed a similar pattern of *GUS* expression in both ifrL–GUS and ifrS–GUS plants. In mature nodules (3 and 5 weeks after inoculation), the majority of the staining was in the proximal nodule meristem (Figure 6F). No staining was observed in the distal meristematic region, the nodule outer cortex, or the region containing the mature, nitrogen-fixing bacteroids. In immature nodules, intense staining was observed across much of the inner meristematic region (Figure 6G). No staining was observed in nodules from plants transformed with
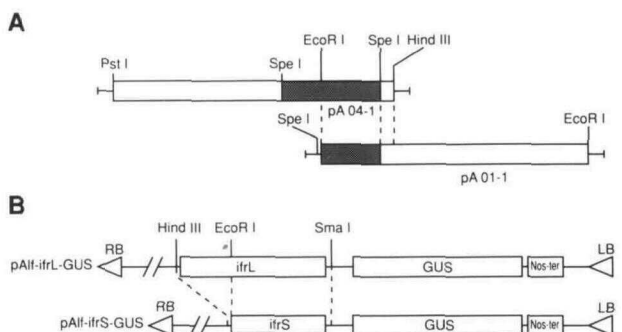
Figure 5. Alfalfa *IFR* Promoter–*GUS* Transcriptional Fusions.

(A) Restriction maps of overlapping subclones pAO1-1 and pAO4-1 from which promoter sequences were isolated. The shaded areas in both clones represent the promoter sequences that were excised by SpeI digestion.

(B) Restriction maps of the binary vectors pAlf-ifrL–GUS and pAlf-ifrS–GUS. RB, right border; LB, left border; Nos-ter, nopaline synthase terminator sequence.

Figure 6. Expression of Alfalfa *IFR* Promoter–*GUS* Fusions in Transgenic Alfalfa.

(A) Roots of alfalfa from an ifrL–GUS plant (×45).
(B) Transverse section of a root from an ifrL–GUS plant (×245).
(C) Transverse section of a root expressing the 35S–*GUS* fusion (×245).
(D) Transverse section of a stem from an ifrL–GUS plant (×45).
(E) Transverse section of a stem expressing the 35S–*GUS* fusion (×100).
(F) Longitudinal section through a mature root nodule from an ifrL–GUS plant (×15).
(G) Longitudinal section through an immature root nodule expressing ifrL–GUS (×23).
(H) Trifoliate leaves taken 7 days after infection of ifrL–GUS plants with *P. medicaginis*. Staining was localized to the immediate region of the lesions.
(I) Trifoliate leaves 7 days after infection of ifrS–GUS plants with *P. medicaginis*.

pBI101.1 or from untransformed control plants. Staining was observed throughout all regions of nodules from plants harboring pBI121.

Inducibility of *IFR* Promoter–*GUS* Fusions in Alfalfa

Transgenic ifrL–GUS and ifrS–GUS plants were inoculated with the alfalfa fungal leaf spot pathogen *Phoma medicaginis.* Only infected leaves showed staining with X-gluc (Figures 6H and 6I). *GUS* expression was very intense in a narrow zone around the lesions. HPLC analysis of portions of leaves similarly infected with *P. medicaginis* revealed that medicarpin accumulation is also confined to this zone (N. Paiva, unpublished results). The staining intensity and pattern were identical with pathogen-infected leaves from both ifrL–GUS and ifrS–GUS plants, and strong staining was observed at 2, 4, and 7 days. Trifoliate leaves of plants transformed with pBI101.1 did not stain after infection with *P. medicaginis,* whereas 35S–GUS plants showed decreased levels of staining on the trifoliate leaves after pathogen infection (data not shown). Wounding did not induce any observable GUS activity.

Transgenic cell suspension cultures (from callus derived from leaves of transgenic alfalfa plants) were treated with a yeast cell wall–derived elicitor that is known to induce strong accumulation of medicarpin (Paiva et al., 1991). Staining with X-gluc was detected only in elicitor-treated cells (data not shown). Using quantitative fluorescence-based GUS assays, an eight- to 13-fold increase in GUS activity was observed in elicited cell cultures derived from ifrS–GUS plants and a similar 10- to 12-fold induction was observed in cultures derived from ifrL–GUS plants (Figure 7). *GUS* expression in cell cultures from 35S–GUS and pBI101-transformed plants was not detectably induced by yeast elicitors. GUS activity in unelicited ifrL– and ifrS–GUS cultures was similar to that in 35S–GUS cultures; constitutive expression of the *IFR* promoter is expected under these conditions, because newly initiated alfalfa cell cultures constitutively accumulate significant amounts of medicarpin-3-O-glucoside-6″-O-malonate (Kessmann et al., 1990b).

Developmental Expression of Alfalfa *IFR* Promoter–*GUS* Fusions in Transgenic Tobacco

The roots of ifrL–GUS tobacco plants had a distinct pattern of X-gluc staining; the strongest staining occurred as a discrete band, excluding the distal end of the root tip (Figure 8A). A longitudinal section through such a root (Figure 8B) showed intense staining in the proximal meristem and high levels of expression throughout the cortex behind this zone. The root cap, the developed vascular tissue, and pith together with the quiescent center and protoderm did not stain. In contrast, expression of *GUS* in tobacco roots controlled by the CaMV 35S promoter included the root tip (Figure 8A). Transverse sections through roots of ifrL–GUS tobacco plants (Figure 8C) showed
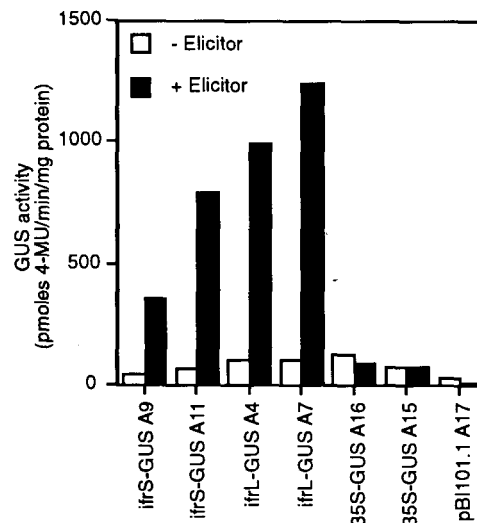

Figure 7. Expression of *IFR* Promoter–*GUS* Fusions in Elicited Alfalfa Cell Suspension Cultures.

Cell suspension cultures derived from transgenic alfalfa plants were treated with yeast elicitor (black bars) or water (white bars) and incubated under standard growth conditions for 17 hr. The designation after the construct name indicates from which independent transgenic line the suspension was derived. Lines harbored the ifrL and ifrS promoter–*GUS* fusions, 35S–*GUS*, or promoterless *GUS* (pBI101.1). GUS activity is given as picomoles of 4-methylumbelliferone (4-MU) per minute per milligram of protein. The value indicated by each bar is the average of two independent GUS enzyme assays; individual measurements for each cell line varied from the mean by no more than ±7% for unelicited and ±2% for elicited cultures. The experiment was repeated with these same cell lines and additional cell lines, and similar levels of expression were obtained.

GUS activity uniformly spanning the entire cortex, with no staining in the epidermis and the central vascular cylinder. *GUS* expression was not detected in the roots of tobacco plants transformed with the promoterless *GUS* construct pBI101.1. In contrast to transgenic alfalfa, no *GUS* expression was observed in the roots of any ifrS–GUS tobacco plants.

Transverse sections through the stems of ifrL–GUS transgenic tobacco plants unexpectedly revealed GUS activity localized to the inner vascular cylinder (Figures 8D and 8E), with parenchyma cells associated with the xylem vessels and primary xylem cells specifically being stained. Figure 8F shows a transverse section through the stem of a tobacco plant transformed with 35S–*GUS.* The heaviest staining with X-gluc is seen through the entire central vascular cylinder in xylem, phloem, and cambial cells. This is similar to the results obtained previously with the 35S promoter–*GUS* fusions in tobacco (Jefferson et al., 1987) and strongly contrasts with the expression pattern of the *IFR* promoter.

Strong X-gluc staining associated with the vascular system was observed in petioles of young leaves (Figure 8G), specifically in the xylem tissue that is sandwiched between two arrays
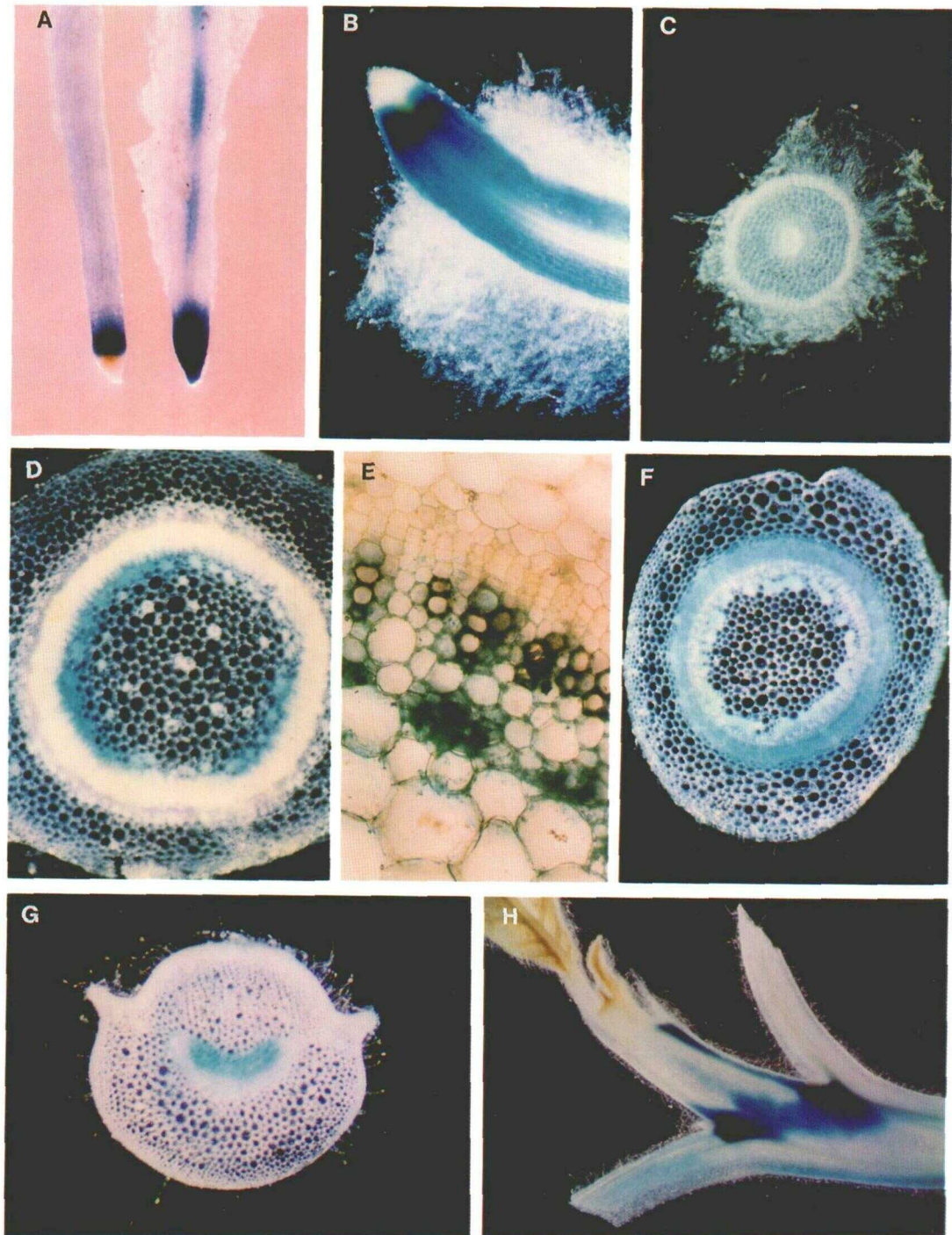
Figure 8. Expression of ifrL–*GUS* Fusions in Vegetative Organs of Transgenic Tobacco.

(A) Comparison of root expression from ifrL–GUS (left) and 35S–GUS (right) plants (×50).

(B) Longitudinal section through a root from an ifrL–GUS plant (×50).

(C) Transverse section through a root from an ifrL–GUS plant (×50).

(D) Transverse section through a stem of an ifrL–GUS plant (×50).

(E) High magnification of a transverse section through a young stem from an ifrL–GUS plant (×55).

(F) Transverse section through a stem of a plant transformed with the 35S–*GUS* fusion (×42).

(G) Transverse section through a petiole of an ifrL–GUS plant (×33).

(H) Longitudinal section through the shoot apex of an ifrL–GUS plant (×8).

The bluish color of the root hairs in **(B)** and **(C)** is a photographic aberration.

of phloem. There was very little or no staining in petioles from the lower one-third of the plant. Expanding leaves expressed little GUS activity; when present, most of the staining was at the leaf tips (data not shown). The area close to lateral emergences such as leaves and branches showed high levels of GUS activity. This is evident in Figure 8H, which shows a longitudinal section through a shoot apex. The heaviest staining was seen at stem peripheries in the regions above the points of attachment of the petioles. The shoot apical meristem shown in Figure 8H did not express any GUS activity. This contrasts with the CaMV 35S promoter, which was functional through-

out the entire shoot apex (data not shown). Unlike the ifrL–GUS plants, GUS activity was not detected in any vegetative organ or cell type of ifrS–GUS tobacco plants. Plants that were generated by transformation with pBl101.1 were also GUS-negative.

GUS activity was observed in various parts of flowers from transgenic ifrL–GUS tobacco plants. In the gynoecium of young flowers, GUS activity was detected in the stigma (Figure 9A) and the placental tissue together with the base of the ovary and the flower receptacle (Figure 9B). The style of mature, open flowers did not stain with X-gluc (Figure 9A), but GUS activity was present in the style of flowers from all earlier stages (data
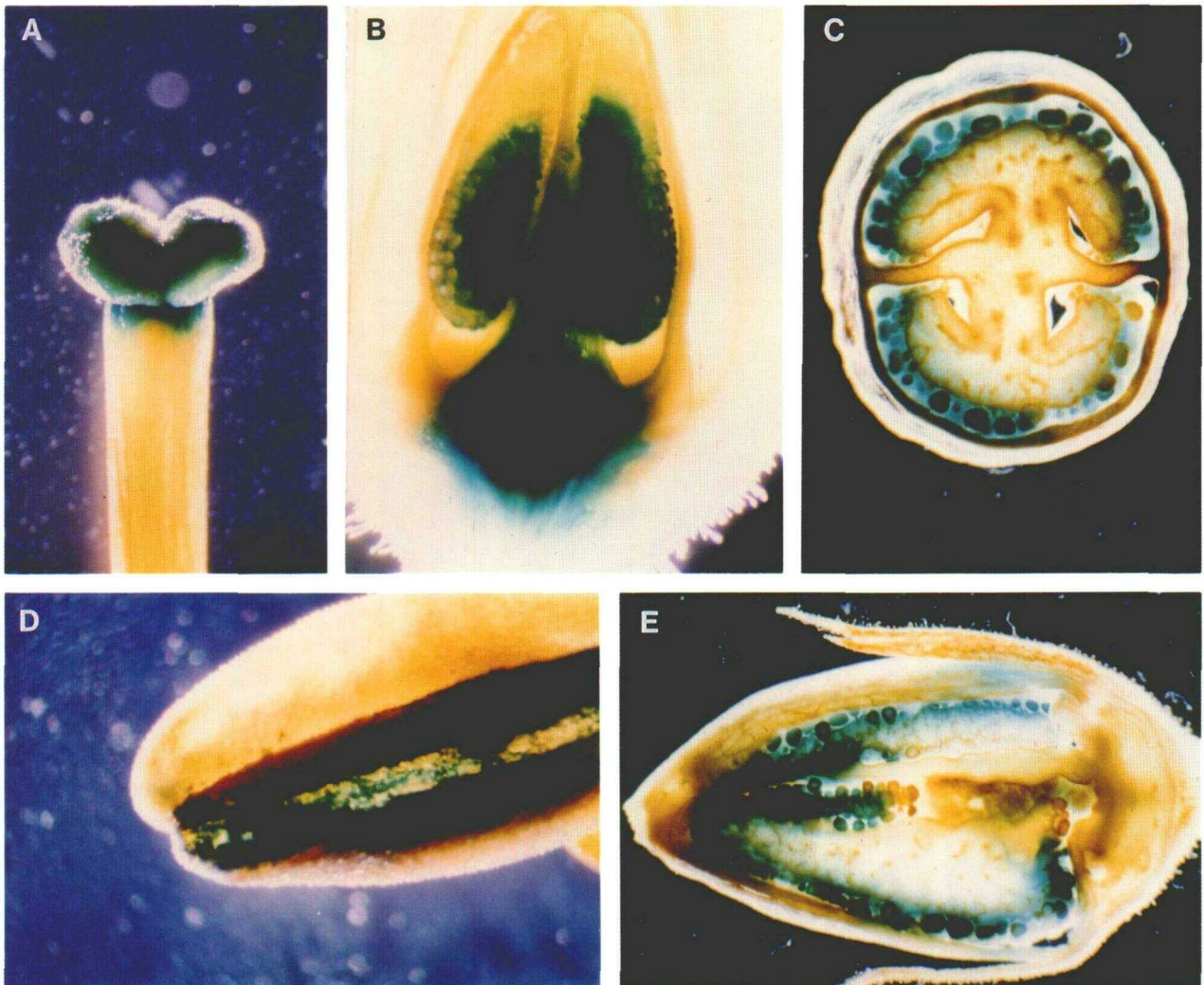
Figure 9. Expression of ifrL–*GUS* and ifrS–*GUS* Fusions in Reproductive Organs of Transgenic Tobacco Plants.

(A) Stigma and style of a mature flower from an ifrL–GUS plant (×30).
(B) Longitudinal section through a mature tobacco flower from an ifrL–GUS plant (×30).
(C) Cross-section through a developing fruit from an ifrL–GUS plant (×30).
(D) Anther of a mature flower of an ifrL–GUS plant showing pollen and extruding tapetal tissue (×60).
(E) Longitudinal section through a developing fruit from an ifrS–GUS plant (×10).

not shown). Ovules from different stages of unopened flowers did not stain, but occasional staining was seen in mature flowers. Whether fertilization is necessary for staining is not known but is a possibility, as cross-sections through maturing fruits (formed after fertilization) showed staining of newly formed seed (Figure 9C). This could be developmentally controlled because there was no staining in the placental region (Figure 9C), which had stained intensely in the mature flower (Figure 9B). In the androecium of tobacco flowers, staining was observed in the tapetal tissue and pollen grains (Figure 9D). Tobacco flowers from all ifrS–GUS plants showed staining in fruits and seed (Figure 9E) and pollen (data not shown). The staining pattern was similar to that of ifrL–GUS flowers (Figures 9C and 9D). No GUS activity was observed in the corolla of flowers of ifrL–GUS or ifrS–GUS plants. In comparison, in tobacco plants harboring the 35S promoter–GUS fusion the entire flower expressed high levels of GUS activity, including the corolla (data not shown). No staining was observed in any part of flowers from plants harboring pBI101, including the pollen.
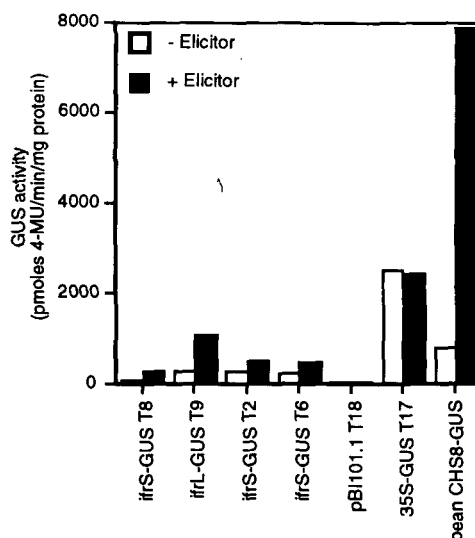
Inducibility of Alfalfa *IFR* Promoter-*GUS* Fusions in Tobacco

Treatment of cell suspension cultures generated from transgenic tobacco plants with yeast elicitor resulted in only a two- to threefold increase in GUS activity for ifrS–GUS cell lines and a three- to fourfold increase in activity over unelicited cells for ifrL–GUS cell lines (Figure 10). This was modest compared with the 10- to 12-fold increase in activity seen upon elicitation of a tobacco cell line transformed with a bean *CHS8–GUS* promoter construct. No consistent increase in GUS activity was detected when transgenic ifrL–GUS and ifrS–GUS tobacco plants were wounded, treated with a variety of tobacco or alfalfa fungal pathogens, infiltrated with *Pseudomonas syringae* pv *syringae* (successfully used to induce the *CHS8* promoter in tobacco; Stermer et al., 1990), treated with salicylic, arachidonic, or jasmonic acids (potential components of defense gene signal transduction pathways; reviewed in Lindsay et al., 1993), or inoculated with tobacco mosaic virus, which forms local lesions on this tobacco strain.

DISCUSSION

Structure of the Alfalfa *IFR* Gene

Previous studies (Paiva et al., 1991) have shown that alfalfa contains a single *IFR* gene, in contrast to the multigene families encoding earlier enzymes of medicarpin synthesis, such as PAL and CHS (Dixon and Harrison, 1990). The very slight differences in nucleotide sequence between the open reading frames of the pIFRalf1 cDNA (Paiva et al., 1991) and the corresponding gene are most likely due to allelic variation

Figure 10. Expression of Alfalfa *IFR* Promoter–*GUS* Fusions in Elicited Tobacco Cell Suspension Cultures.

Cell suspension cultures derived from transgenic tobacco plants were treated with yeast elicitor (black bar) or with water (white bar) and incubated under standard growth conditions for 17 hr. The plants from which the cell lines were derived are indicated. CHS8 is a tobacco line that harbors the bean *CHS8* promoter fused to *GUS* (Stermer et al., 1990). The tobacco line pBI101.1 T18 harbors the promoterless *GUS* construct pBI101.1. GUS activity is expressed in picomoles of 4-methylumbelliferone (4-MU) per minute per milligram of protein. The value indicated by each bar is the average of two independent GUS enzyme assays; individual measurements for each cell line varied from the mean by no more than ±4%.

among individuals in the highly heterogeneous cultivar Apollo; unlike other species, alfalfa cultivars are actually blends of progeny from hundreds of intercrossed parents.

The 954-bp open reading frame of the *IFR* gene is interrupted by four short introns. Most *PAL* and *CHS* genes reported to date contain a single intron, whereas the gene encoding dihydroflavonol-4-reductase from Antirrhinum and petunia, which shares significant sequence similarity with *IFR* at the N terminus, contains five introns (Beld et al., 1989). A tobacco cDNA termed TP7, corresponding transcripts of which are expressed specifically in flower limb tissue (Drews et al., 1992), exhibits high nucleotide sequence identity with *IFR* in the first through fifth exons (48.3, 62.2, 64.4, 45.1, and 54.1%, respectively) (T.P. Beals and R.B. Goldberg, unpublished data). The N-terminal region of TP7 contains a putative NADPH binding site similar to that found in *IFR* (Paiva et al., 1994b). It is possible that reductase genes involved in branch pathways of flavonoid/isoflavonoid biosynthesis have evolved from combinations of exonic structure–function modules (Traut, 1988).

The 765 bp of available *IFR* promoter sequence were searched for sequences similar to previously identified *cis* elements functional in stress-induced or developmentally regulated expression of plant defense genes. Best matches

were for near complete (−620) and partial (−140) elements homologous to the box P region identified as important for elicitor responsiveness of a parsley *PAL* promoter (Lois et al., 1989). An alignment of these conserved sequences is shown in Figure 11. Box P binds an elicitor-inducible basic leucine zipper transcription factor termed BPF-1 (da Costa e Silva et al., 1993), and related sequences are also present in bean *PAL* and *CHS* genes (Dixon and Harrison, 1990) and in the alfalfa *CHS2* gene (N.L. Paiva, H. Junghans, R.A. Gonzales, and R.A. Dixon, unpublished data). Other important elements such as the H-box, G-box, and SBF-1 binding sites, which have been implicated in elicitor-mediated and developmental expression of bean *CHS* genes (Harrison et al., 1991; Loake et al., 1992; Yu et al., 1993), were not present in the *IFR* upstream region.

The Alfalfa *IFR* Gene Is Transcriptionally Activated in Elicited Cells

Nuclear transcription run-on analyses have confirmed transcriptional activation of several plant defense response genes, including genes encoding the phenylpropanoid pathway enzymes PAL, 4-coumarate:coenzyme A ligase, CHS, and chalcone isomerase, in elicited cells (Dixon and Harrison,

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                      A   A       C
Box P consensus    C C A   C   A A C   C C
                          C   T       T

Pc PAL -193    C T C C A A C A A A C C C C T

Ms IFR -620        C C A C C T A A C A T C

Ms IFR -208        T T A A C T G A C T A C

Ms IFR -140        C C A A C A G G G C T G


                   T   C           C   C
Box L consensus      C T   A C C T A   C
                   A   A               A   A

Pc PAL -119    A T T C T C A C C T A C C A

Ms IFR -622        I A C C A C C T A A C A

Ms IFR -210        I T I T A A C T G A C T

Ms IFR -146        I T I T G A C C A A C A
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Figure 11. Putative Elements of the *IFR* Promoter That May Be Conserved in Other Phenylpropanoid Pathway Genes.

Portions of the *Medicago sativa* (Ms) *IFR* promoter sequence were aligned with box P and box L consensus sequences and the regions displaying elicitor and UV-induced footprints in the *Petroselinum crispum* (Pc) *PAL* gene (Lois et al., 1989). Bases in the *IFR* promoter sequence that match the consensus sequence are underlined. Both box P and box L consensus sequences can be aligned with the same regions of the *IFR* promoter because the two consensus sequences can be aligned with each other (bases 2 to 10 of the box P consensus align with bases 4 to 12 of the box L consensus).

1990). In parsley cells, the kinetics of transcriptional activity of a range of elicitor-induced genes exhibited considerable variation, suggesting multiple mechanisms for defense gene activation (Somssich et al., 1989). In our study, the transcription kinetics of PAL, the first enzyme in the phytoalexin pathway, and IFR, the penultimate enzyme, were broadly similar, suggesting that activation of PAL is not a prerequisite for induction of downstream enzymes. A detailed analysis of the very early activation kinetics (0 to 60 min) of medicarpin pathway genes has revealed nearly identical patterns for several of the genes examined, including those encoding PAL, CHS, and IFR (W. Ni and R.A. Dixon, unpublished data).

cis Elements Downstream of −436 Are Sufficient for Developmental and Elicitor-Induced Expression of the *IFR* Promoter in Alfalfa

RNA gel blot analysis (Paiva et al., 1991) and in situ hybridization studies (Harrison and Dixon, 1994) have shown that IFR transcripts are constitutively expressed in nodules and root cortical cells of alfalfa plants. This correlates with the accumulation of medicarpin malonyl glycoside in root tissues and the histochemical localization of isoflavonoid accumulation in the root cortex of legumes with similar medicarpin conjugate accumulation (Wiermann, 1981). The absence of IFR transcripts in the aerial organs of uninfected plants likewise reflects the absence of isoflavonoid compounds in these organs. The expression patterns observed with −765 and −436 *IFR* promoter–*GUS* fusions in transgenic alfalfa indicate that the promoter confers correct developmental expression in the homologous species and that root/nodule expression is determined by sequences downstream of −436. It is not known whether the distinctive lack of GUS staining in the region distal to the root apical meristem and cell proliferation zone reflects differences in isoflavonoid metabolism in the meristematic and quiescent regions of the root tip. The bean *PAL2* promoter exhibits strong expression in the cell proliferation zone immediately adjacent to the root apical meristem in transgenic tobacco (Liang et al., 1989). In addition, the bean *CHS8* promoter (Schmid et al., 1990) is also expressed in this zone, in the root apical meristem, and in emerging lateral roots in a manner similar to that observed for the *IFR* promoter. It has been suggested that phenylpropanoid/flavonoid synthesis at the root apex may produce morphogenetic signals affecting polar auxin transport or exhibiting cytokinin-like activity (Liang et al., 1989) based on observations of biological activities of flavonoids (Jacobs and Rubery, 1988) and dehydrodiconiferyl glucosides (Binns et al., 1987), respectively. Whether isoflavonoids have morphogenetic activity or whether their synthesis at the root tip is related to rhizosphere colonization remains to be determined.

Alfalfa nodules are indeterminant and maintain an active meristematic region under normal conditions (Hirsch et al., 1992). The location of strongest *GUS* expression in nodules is in a tissue analogous to the strongest staining tissue in root

tips, namely the proximal nodule meristem. Unlike many other legumes, alfalfa constitutively accumulates significant amounts of phytoalexin conjugates in roots and effective nodules (Paiva et al., 1991). It is thought that either the *R. meliloti* symbiont is not sensitive to medicarpin (Pankhurst and Biggs, 1980) or the conjugated form, or the conjugation keeps the phytoalexin sequestered in the plant cell vacuoles away from the symbiont. We observed no evidence of promoter activity in the cells that contain the bacteroids, the actual N_2-fixing form of the symbiont, suggesting that phytoalexin biosynthesis is not occurring in these cells.

In transgenic alfalfa suspension cultures, the *IFR* promoter is strongly activated (up to 13-fold increase in GUS activity) in response to treatment with yeast elicitor, with basal expression in unelicited cells being equivalent to that of the constitutive 35S promoter. This increase in GUS activity is similar to the increase in IFR activity (10- to 12-fold) observed in elicited alfalfa cells (Paiva et al., 1991). As elicitor responsiveness is not lost on deletion to −436, the putative box P–like element between positions −620 and −609 cannot be required for reception of the elicitation stimulus. Other than an incomplete box P core sequence (CCAACA) at position −140 and some scattered homology near position −210 (see Figure 11), there are no sequence elements common to other elicitor-induced phytoalexin biosynthetic genes in the *IFR* promoter downstream of position −436. This suggests that, although *IFR* transcription is activated simultaneously with that of other defense genes, separate signaling pathways may exist for the activation of early- and late-pathway genes in response to elicitors.

The Major Developmental Expression Pattern of the *IFR* Promoter in Transgenic Tobacco Is Controlled by Sequences Upstream of −436

Tobacco does not possess the isoflavonoid branch of phenylpropanoid biosynthesis. Most previous studies in which correct developmental expression of a transgene has been reported in a heterologous species have involved promoters of genes whose products are common to both source and recipient of the transgene (Benfey and Chua, 1989). The alfalfa *IFR* promoter is expressed in stem and floral tissues of tobacco according to a developmental program different from that seen in alfalfa. This difference is particularly striking in terms of the strong expression in tobacco stem and floral tissue. Although some features of this expression are similar to those of bean *PAL* and *CHS* transgenes and tobacco *IFR*-like TP7 transcripts in tobacco (Liang et al., 1989; Schmid et al., 1990; Drews et al., 1992), there is a notable absence of *IFR* promoter expression in petal tissue. Furthermore, the elicitor responsiveness of the promoter in transgenic tobacco cell suspensions was very poor compared with that of the bean *CHS8* promoter (Figure 10). It is therefore possible that the *IFR* promoter is responsive to signals regulating the developmental programs of more than one differentially expressed endogenous tobacco gene that may or may not have defensive roles. The high constitutive

expression in the above-ground parts of tobacco may also be due in part to the lack of specific negative regulatory factors in tobacco, which might normally suppress expression in these parts of alfalfa plants.

Although the 756-bp *IFR* promoter conferred similar patterns of *GUS* expression in cortical cells and root meristems of tobacco and alfalfa, the *cis* elements conferring this expression are different in the two cases, as deletion to −436 abolishes root expression in tobacco but not in alfalfa. Identification of the sequences conferring root specificity must await a detailed mutational and deletional analysis of the promoter.

During the analysis of promoters from several nonsolanaceous, "nontransformable" species, investigators have relied on the use of promoter–deletion/reporter gene fusions expressed in tobacco to identify putative regulatory *cis* elements (e.g., see Liang et al., 1989; Schmid et al., 1990; Fritze et al., 1991; Burnett et al., 1993; Mason et al., 1993). Our results indicate that data from such heterologous transformation experiments may be misleading. Constructs that yield ectopic expression (such as in stems, flowers, and seed of ifrL–GUS plants) or that appear inactive (such as in roots of ifrS–GUS plants) in tobacco may exhibit correct developmental and stress-induced expression in the homologous system.

Implications for Use of the *IFR* Promoter in Biotechnology

Under inductive conditions in alfalfa cell cultures, the *IFR* promoter confers stronger expression on a reporter gene than the commonly used constitutive 35S promoter. These properties suggest that it may be a useful tool for engineering resistance responses, at least in the important forage legume alfalfa. Constitutive root expression of nonphytotoxic defense gene products could be advantageous in some cases, as would lack of constitutive expression in aerial parts of the forage. It may be possible to delete or alter portions of the *IFR* promoter to eliminate the constitutive root expression, while retaining localized pathogen inducibility, as was achieved for the tobacco *TobRB7* promoter (Opperman et al., 1994). Such a pathogen-specific promoter would be valuable in defense strategies in which both the pathogen and a few host cells are killed by the localized production of a highly toxic product. The promoter may also be useful in screening for substances or conditions that induce defense reactions (Doerner et al., 1990) or as an early indicator of plant infection. Current studies do, however, indicate the dangers inherent in assuming that defense-responsive promoters will function predictably in heterologous environments.

METHODS

Plant Material

vegetative growth and induction of flowering, alfalfa plants were maintained at 18 hr/25°C day and 6 hr/19°C night cycles at ~70% relative humidity in controlled environmental chambers (Conviron, Winnipeg, Manitoba, Canada). Tobacco plants were maintained in the greenhouse at 16-hr/25°C day and 8-hr/20°C night cycles at ~50% relative humidity.

Callus and cell suspension cultures of alfalfa and tobacco were generated and maintained on modified SH media as described by Kessmann et al. (1990a). Yeast cell wall preparations were used to elicit cell cultures as described previously (Paiva et al., 1991).

Nodulation and Pathogen Infection of Alfalfa

Multiple cuttings of transgenic plants were rooted in autoclaved perlite wetted with filter-sterilized nitrogen-free Hoagland's nutrient solution supplemented with trace elements. After roots formed (10 days), 5 mL of a suspension of *Rhizobium meliloti* 102F51 (OD_{600} of 0.5) was added to each cutting. Sterile water or nutrient solution was added as necessary to prevent the perlite from drying out. Nodulated roots were examined 9 days, 3 weeks, and 5 weeks after inoculation.

Growth chamber–grown transgenic plants were sprayed either with a spore suspension (~10^5 colony-forming units per mL) of *Phoma medicaginis* in 0.05% Tween-20 or with dilute Tween-20 alone. Inoculated and control plants were then enclosed in clear plastic bags and grown under high humidity. Trifoliate leaves were taken at intervals of 2, 4, and 7 days and stained with 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) to detect β-glucuronidase (GUS) activity.

Isolation of Genomic Clones

A genomic library of the alfalfa cultivar Apollo (constructed by G. Gowri and B. Shorrosh, Noble Foundation) in the λFix II system (Stratagene) was screened using as a probe the 0.75-kb internal HindIII fragment of pIFRalf1 (Paiva et al., 1991). Hybridization and washing conditions were as recommended in the manual (preferred method) supplied with the Colony/Plaque Screen hybridization transfer membranes (Du Pont) except that 6 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) replaced 1 M NaCl in the hybridizations and 2 × SSPE replaced 2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate) in the washes after hybridizations. Positive clones from the first round of screening were purified by two additional rounds of screening. DNA from purified phage clones was analyzed by restriction endonuclease digestion and DNA gel blot hybridization; selected fragments were subcloned into pBluescript II SK– for sequencing and further analysis.

DNA and RNA Gel Blot Hybridization

DNA was isolated from tobacco and alfalfa plants as described previously (Junghans and Metzlaff, 1990). RNA was isolated as described by Paiva et al. (1991). Up to 10 μg of total RNA or genomic DNA was used in RNA or DNA gel blot analysis, respectively. Transfer of DNA or RNA to nylon membranes and hybridizations to specific probes were performed as recommended in the manual (preferred method) supplied with GeneScreen Plus hybridization transfer membranes (Du Pont); changes to the hybridization and washing conditions were as described above for library screening. Probes were labeled to high specific activity by random primer labeling (Feinberg and Vogelstein, 1984).

DNA Sequence Analysis

DNA was sequenced by the Sanger dideoxy sequencing method (Sanger et al., 1977). A *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's protocol. The products were separated on a 6% polyacrylamide gel, and the data were processed by an ABI 373A automated DNA sequencer (Applied Biosystems). All manipulations of raw data generated from the automated system were done on the PC Gene DNA analysis software (IntelliGenetics, Mountain View, CA).

Primer Extension Analysis

A synthetic 22-mer oligonucleotide complementary to the N-terminal end of the isoflavone reductase (*IFR*) coding region (positions 93 to 114; GGATTTTGTTTTCAGTTGCCAT) was end labeled with T4 polynucleotide kinase and γ-^{32}P-ATP. Up to 5 × 10^4 cpm of labeled primer was annealed to 10 μg of total RNA in a 20-μL reaction containing 100 mM Tris-HCl, pH 8.3, 140 mM KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 1 mM deoxynucleotide triphosphates, and 40 units of RNasin (Promega) (Pfitzner et al., 1988). The reaction was performed in the presence of 200 units of reverse transcriptase (Superscript, Bethesda Research Laboratories) at 42°C for 45 min. The products of the primer extension reaction were analyzed on a 6% polyacrylamide gel containing 8 M urea along with a sequencing reaction done on pAO1-1 with the same primer. Sequencing for the primer extension reaction was done using Sequenase (U.S. Biochemical Corp.) according to the instructions provided by the vendor.

Run-On Transcription

Nuclei were isolated from frozen elicited alfalfa cells, and run-on transcription and transcript isolation procedures were performed as described by Ni and Trelease (1991). Slot blots were prepared using GeneScreen Plus hybridization membranes with 1 μg of DNA per lane; the probe consisted of the 0.75-kb HindIII fragment of pIFRalf1.

IFR Promoter–*GUS* Fusions

Plasmids pAO1-1 and pAO4-1 contain overlapping regions of the IFR genomic clone (see Results). Digestion of the plasmid pAO1-1 yielded a 540-bp SpeI fragment containing 436 bp of the promoter region (EcoRI to SpeI site) and 80 bp of the 5' untranslated region, along with 24 bases of the vector's multiple cloning sites. Digestion of pAO4-1 yielded a 845-bp SpeI fragment containing an additional 329 bp of upstream promoter sequence. These two SpeI fragments were ligated into the XbaI site of pBI101.1 (Jefferson et al., 1987) to generate pAlf-ifrS–GUS and pAlf-ifrL–GUS, respectively. The two binary vectors were maintained in *Escherichia coli* DH5α and then transferred to *Agrobacterium tumefaciens* LBA4404 by direct DNA transfer (An, 1987).

Plant Transformation and Regeneration

Tobacco and alfalfa plants were transformed with Agrobacterium strain LBA4404 harboring the gene construct of interest by leaf disc methods. Transgenic tobacco plants (*Nicotiana tabacum* cv Xanthi) were generated as described previously (Rogers et al., 1986), with regeneration under kanamycin selection. Transgenic alfalfa plants were generated from the transformation- and regeneration-competent alfalfa cultivar

Regen SY (Bingham, 1991), following a modified version of published procedures (Bingham et al., 1975). Briefly, pieces cut from young leaves were inoculated with a suspension of Agrobacterium harboring the binary construct and incubated on solid B5h (Brown and Atanassov, 1985) plates for 4 days (16 hr of light at 24°C). The explants were washed twice with water to remove bacteria and incubated for 4 more days on new B5h plates. Explants were then washed twice with water and transferred to selection plates (B5h plates with 100 mg/L timentin [Beecham Inc., Bristol, TN] and 25 mg/L kanamycin [Sigma]). Calli and a few embryos appeared after 2 weeks and were transferred to new selection plates, making sure the calli were spread out. Plants were incubated for another week to allow the development of additional embryos. The calli and embryos were then transferred to B5 plates (no hormones but with antibiotics as before). After 2 weeks, the calli and embryos were transferred to fresh B5 plates (with antibiotics). After 1 to 2 weeks, individual embryos were cultured on Murashige and Skoog (MS; Murashige and Skoog, 1962) plates with antibiotics (50 mg/L timentin and 25 mg/L kanamycin); plantlets were formed within 1 to 3 weeks, sometimes with roots. These were transferred to plastic boxes (Magenta Corp., Chicago, IL) with MS agar media and antibiotics. Plants were maintained on MS media with antibiotics and propagated by cutting.

Histochemical Localization and Fluorometric Quantitation of GUS Activity

GUS activity was localized histochemically by standard protocols (Jefferson, 1987; Martin et al., 1992). Typically, sectioned tissues or whole plant parts were incubated in 75 mM sodium phosphate, pH 7.5, 1 to 2 mM X-gluc in 10% dimethylformamide, and 0.5% Triton X-100 for 6 to 12 hr at 37°C. The fluorescence of 4-methylumbelliferone produced by cleavage of 4-methylumbelliferyl β-D-glucuronic acid was measured to quantitate GUS activity (Jefferson, 1987), which was expressed as picomoles of 4-methylumbelliferone produced per minutes per milligram of protein. Protein concentration was measured by the Bradford assay (Bradford, 1976) using the Bio-Rad protein assay reagent.

REFERENCES

An, G. (1987). Binary Ti vectors for plant transformation and promoter analysis. Methods Enzymol. 153, 292-305.

Beld, M., Martin, C., Huits, H., Stuitje, A.R., and Gerats, A.G.M. (1989). Flavonoid synthesis in Petunia hybrida: Partial characterization of dihydroflavonol-4-reductase genes. Plant Mol. Biol. 13, 491-502.

Benfey, P.N., and Chua, N.-H. (1989). Regulated genes in transgenic plants. Science 244, 174-181.

Bingham, E.T. (1991). Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. Crop Sci. 31, 1098.

Bingham, E.T., Hurley, L.V., Kaatz, D.M., and Saunders, J.W. (1975). Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci. 15, 719-721.

Binns, A.N., Chen, R.H., Wood, M.N., and Lynn, D.G. (1987). Cell division promoting activity of naturally occurring dehydrodiconiferyl glucosides: Do cell wall components control cell division? Proc. Natl. Acad. Sci. USA 84, 615-619.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Breathnach, U., and Chambon, P. (1981). Organization and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. 50, 349-384.

Brown, O.C.W., and Atanassov, A. (1985). Role of genetic background in somatic embryogenesis in Medicago. Plant Cell Tissue Organ Cult. 4, 111-122.

Burnett, R.J., Maldonado-Mendoza, I.E., McKnight, T.D., and Nessler, C.L. (1993). Expression of a 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from Camptotheca acuminata is differentially regulated by wounding and methyl jasmonate. Plant Physiol. 103, 41-48.

da Costa e Silva, O., Klein, L., Schmelzer, E., Trezzini, G., and Hahlbrock, K. (1993). BPF-1, a pathogen-induced DNA-binding protein involved in the plant defense response. Plant J. 4, 125-135.

Dewick, P.M. (1988). Isoflavonoids. In The Flavonoids: Advances in Research Since 1980, J.B. Harborne, ed (New York: Chapman and Hall), pp. 125-210.

Dixon, R.A., and Harrison, M.J. (1990). Activation, structure and organization of genes involved in microbial defense in plants. Adv. Genet. 28, 165-234.

Dixon, R.A., Dey, P.M., and Lamb, C.J. (1983). Phytoalexins: Enzymology and molecular biology. Adv. Enzymol. Relat. Areas Mol. Biol. 55, 1-136.

Doerner, P.W., Stermer, B., Schmid, J., Dixon, R.A., and Lamb, C.J. (1990). Plant defense gene promoter-reporter gene fusions in transgenic plants: Tools for identification of novel inducers. Bio/Technology 8, 845-848.

Drews, G.N., Beals, T.P., Bui, A.Q., and Goldberg, R.B. (1992). Regional and cell-specific gene expression patterns during petal development. Plant Cell 4, 1383-1404.

Feinberg, A.P., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to a high specific activity. Anal. Biochem. 137, 266-267.

Fritze, K., Staiger, D., Czaja, I., Walden, R., Schell, J., and Wing, D. (1991). Developmental and UV light regulation of the snapdragon chalcone synthase promoter. Plant Cell 3, 893-905.

Harrison, M.J., and Dixon, R.A. (1994). Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of Medicago truncatula and the mycorrhizal fungus Glomus versiforme. Plant J. 6, 9-20.

Harrison, M.J., Lawton, M.A., Lamb, C.J., and Dixon, R.A. (1991). Characterization of a nuclear protein that binds to three elements within the silencer region of a bean chalcone synthase gene promoter. Proc. Natl. Acad. Sci. USA **88**, 2515–2519.

Higgins, V.J. (1972). Role of the phytoalexin medicarpin in three leaf spot diseases of alfalfa. Physiol. Plant Pathol. **2**, 289–300.

Hirsch, A.M., McKhann, H.I., and Löbler, M. (1992). Bacterial-induced changes in plant form and function. Int. J. Plant Sci. **153** (suppl.), 171–181.

Jacobs, M., and Rubery, P.H. (1988). Naturally occurring auxin transport regulators. Science **241**, 346–349.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant. Mol. Biol. Rep. **5**, 387–405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901–3907.

Junghans, H., and Metzlaff, M. (1990). A simple and rapid method for the preparation of total plant DNA. BioTechniques **8**, 176.

Kessmann, H., Choudhary, A.D., and Dixon, R.A. (1990a). Stress responses in alfalfa (*Medicago sativa* L.). III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts. Plant Cell Rep. **9**, 38–41.

Kessmann, H., Edwards, R., Geno, P., and Dixon, R.A. (1990b). Stress responses in alfalfa (*Medicago sativa* L.). V. Constitutive and elicitor-induced accumulation of isoflavonoid conjugates in cell suspension cultures. Plant Physiol. **94**, 227–232.

Kuć, J. (1982). Phytoalexins from the Solanaceae. In Phytoalexins, J.A. Bailey and J.W. Mansfield, eds (New York: Halsted Press), pp. 81–105.

Liang, X., Dron, M., Schmid, J., Dixon, R.A., and Lamb, C.J. (1989). Developmental and environmental regulation of a phenylalanine ammonia-lyase-β-glucuronidase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **86**, 9284–9288.

Lindsay, W.P., Lamb, C.J., and Dixon, R.A. (1993). Microbial recognition and activation of plant defense systems. Trends Microbiol. **1**, 181–187.

Loake, G.J., Faktor, O., Lamb, C.J., and Dixon, R.A. (1992). Combination of H-box [CCTACC(N)₇CT] and G-box (CACGTG) *cis* elements is necessary for feed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid-pathway intermediate *p*-coumaric acid. Proc. Natl. Acad. Sci. USA **89**, 9230–9234.

Lois, R., Dietrich, A., Hahlbrock, K., and Schulz, W. (1989). A phenylalanine ammonia-lyase gene from parsley: Structure and identification of elicitor and light responsive *cis*-acting elements. EMBO J. **8**, 1641–1648.

Martin, T., Schmidt, R., Altmann, T., and Frommer, W.B. (1992). Non-destructive assay systems for detection of β-glucuronidase activity in higher plants. Plant Mol. Biol. Rep. **10**, 37–46.

Mason, H.S., DeWald, D.B., and Mullet, J.E. (1993). Identification of a methyl jasmonate–responsive domain in the soybean *vspB* promoter. Plant Cell **5**, 241–251.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473–497.

Ni, W., and Trelease, R.N. (1991). Post-transcriptional regulation of catalase isozyme expression in cotton seeds. Plant Cell **3**, 737–744.

Ohl, S., Hedrick, S.A., Chory, J., and Lamb, C.J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell **2**, 837–848.

Opperman, C.H., Taylor, C.G., and Conkling, M.A. (1994). Root-knot nematode–directed expression of a plant root-specific gene. Science **263**, 221–223.

Paiva, N.L., Edwards, R., Sun, Y., Hrazdina, G., and Dixon, R.A. (1991). Stress responses in alfalfa (*Medicago sativa* L.). 11. Molecular cloning and expression of alfalfa isoflavone reductase, a key enzyme of isoflavonoid phytoalexin biosynthesis. Plant Mol. Biol. **17**, 653–667.

Paiva, N.L., Oommen, A., Harrison, M.J., and Dixon, R.A. (1994a). Regulation of isoflavonoid metabolism in alfalfa. Plant Cell Tissue Organ Cult., in press.

Paiva, N.L., Sun, Y., Dixon, R.A., VanEtten, H.D., and Hrazdina, G. (1994b). Molecular cloning of isoflavone reductase from pea (*Pisum sativum* L.): Evidence for a 3R-isoflavanone intermediate in (+)-pisatin biosynthesis. Arch. Biochem. Biophys. **312**, 501–510.

Pankhurst, C.E., and Biggs, D.R. (1980). Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Microbiol. **26**, 542–545.

Pfitzner, U., Pfitzner, A., and Goodman, H.M. (1988). DNA sequence analysis of a PR-1a gene from tobacco: Molecular relationship of heat shock and pathogen responses in plants. Mol. Gen. Genet. **211**, 290–295.

Rogers, S.G., Horsch, R.B., and Fraley, R.T. (1986). Gene transfer in plants: Production of transformed plants using Ti plasmid vectors. Methods Enzymol. **118**, 627–640.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**, 5463–5467.

Schmid, J., Doerner, P.W., Clouse, S.D., Dixon, R.A., and Lamb, C.J. (1990). Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell **2**, 619–631.

Somssich, I.E., Bollmann, J., Hahlbrock, K., Kombrink, E., and Schulz, W. (1989). Differential early activation of defense-related genes in elicitor-treated parsley cells. Plant Mol. Biol. **12**, 227–234.

Stermer, B.A., Schmid, J., Lamb, C.J., and Dixon, R.A. (1990). Infection and stress activation of bean chalcone synthase promoters in transgenic tobacco. Mol. Plant-Microbe Interact. **3**, 381–388.

Tiemann, K., Inzé, D., Van Montagu, M., and Barz, W. (1991). Pterocarpan phytoalexin biosynthesis in elicitor-challenged chickpea (*Cicer arietinum* L.) cell cultures: Purification, characterization and cDNA cloning of NADPH:isoflavone oxidoreductase. Eur. J. Biochem. **200**, 751–757.

Traut, T.W. (1988). Do exons code for structural or functional units in proteins? Proc. Natl. Acad. Sci. USA **85**, 2944–2948.

Wiermann, R. (1981). Secondary plant products and cell and tissue differentiation. In The Biochemistry of Plants, Vol. 7, P.K. Stumpf and E.E. Conn, eds (New York: Academic Press), pp. 85–116.

Yu, L.M., Lamb, C.J., and Dixon, R.A. (1993). Purification and biochemical characterization of proteins which bind to the H-box *cis*-element implicated in transcriptional activation of plant defense genes. Plant J. **3**, 805–816.