

Stress Activation of a Bean Hydroxyproline-Rich Glycoprotein Promoter Is Superimposed on a Pattern of Tissue-Specific Developmental Expression¹

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The HRGP4.1 gene, which encodes a cell wall hydroxyproline-rich glycoprotein, was isolated from a genomic library of bean (*Phaseolus vulgaris* L.). Two transcripts, one induced by wounding and one by elicitation, were transcribed from the same initiation site. The gene encodes a polypeptide of 580 amino acids with the amino terminal half consisting of repeats of the sequence serine-(proline)₄-lysine-histidine-serine-(proline)₄-(tyrosine)₃-histidine and the carboxyl-terminal half composed of repeats of the sequence serine-(proline)₄-valine-tyrosine-lysine-tyrosine-lysine. A 964-bp upstream promoter fragment was translationally fused to the β -glucuronidase reporter gene (*Escherichia coli uidA*) and transferred into tobacco by *Agrobacterium tumefaciens*-mediated leaf disc transformation. Analysis of β -glucuronidase activity showed that wounding caused local activation of the HRGP4.1 promoter in the phloem. Infection by tobacco mosaic virus was a less effective inducer than wounding. Stress induction was superimposed on tissue-specific developmental expression in stem nodes and root tips, suggesting that HRGP4.1 may have specific structural roles in development as well as protective functions in defense. Deletion analysis showed that control of tissue specificity and wound inducibility lies in a region between -94 and -251 relative to the transcription start site and that activation by infection lies outside that region.

A family of HRGPs constitutes a major portion of the protein component of plant cell walls (Varner and Lin, 1989; Wycoff et al., 1992; Showalter, 1993; Kieliszewski and Lamport, 1994). The amino acid sequences of these cell wall HRGPs, or extensins, are highly repetitive, containing ar-

rays of the pentapeptide sequence Ser-(Hyp)₄, often within a higher-order repeat motif. Oligo-arabinosides are attached by O-glycosidic bonds to the Hyp residues (Varner and Lin, 1989), which are generated by posttranslational hydroxylation of Pro. The carbohydrate is thought to be important in maintaining the structure of HRGPs as linear, rod-like molecules (Stafstrom and Staehelin, 1988). HRGPs accumulate as soluble cell wall proteins at certain stages of development and eventually become insolubilized, putatively by the formation of intermolecular cross-linkages (Cooper and Varner, 1983; Biggs and Fry, 1990; Kieliszewski and Lamport, 1994).

The deposition and cross-linking of these abundant rod-like molecules have been proposed to increase the mechanical strength of the cell wall (Varner and Lin, 1989). Immunohistochemical experiments have shown that a soybean HRGP is localized in the sclerenchyma tissue of the seed coat and in the vascular tissue of cotyledons (Cassab and Varner, 1987). This localization is consistent with a putative function in the structural reinforcement of the wall of specific cell types. HRGPs and HRGP RNAs also accumulate in response to wounding (Corbin et al., 1987; Sauer et al., 1990), treatment with microbial elicitors (Showalter et al., 1985; Corbin et al., 1987), and pathogen infection (Leach et al., 1982; Hammerschmidt et al., 1984; Showalter et al., 1985; Mazau and Esquerré-Tugayé, 1986; Corbin et al., 1987; Benhamou et al., 1990). Stress-induced HRGPs may provide a structural barrier against infection directly or by providing sites for lignin deposition (Hammerschmidt et al., 1984). In certain cases they may function as specific microbial agglutinins (Leach et al., 1982; Mellon and Helgeson, 1982).

HRGPs are encoded by a family of genes (Showalter, 1993), and we are studying their structure and expression as an approach to elucidating the molecular mechanisms that govern the functional architecture of the plant cell wall in the differentiation of specific cell types and in response to environmental stress. Evidence indicates that the selective transcriptional activation of specific HRGP genes de-

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Abbreviations: HRGP, Hyp-rich glycoprotein; nt, nucleotide; PAL, Phe ammonia-lyase; TMV, tobacco mosaic virus; X-Gluc, 5-bromo-4-chloro-3-indoyl- β -D-glucopyranoside.

termines the pattern of deposition of HRGP structural variants during development. For instance, the promoter of a tobacco HRGP gene, HRGPnt3, directed a precise and limited pattern of expression during development (Keller and Lamb, 1989). This promoter was active only in a subset of endodermal and pericycle cells involved in the initiation of a lateral root. It was not activated in surrounding tissues of the primary root damaged during lateral root initiation or in response to externally imposed wounding. The exquisite specificity of HRGPnt3 expression implies a correspondingly specialized function of the encoded protein, possibly in the strengthening of the lateral root tips to withstand the pressure arising from mechanical penetration through surrounding tissues of the primary root.

To investigate this concept of selective transcriptional activation in relation to HRGP function in defense, we have isolated an HRGP gene, HRGP4.1, from bean (*Phaseolus vulgaris* L.) that corresponds to a previously characterized stress-inducible cDNA, Hyp4.1 (Corbin et al., 1987). The Hyp4.1 cDNA was isolated from a cDNA library made from elicitor-induced cells (Corbin et al., 1987) and encodes a Tyr-rich HRGP. Hyp4.1 hybridizes to a transcript of 2.5 kb that accumulates in cells treated with fungal elicitor and hypocotyls inoculated with the fungal pathogen *Colletotrichum lindemuthianum*. Hyp4.1 also hybridizes to a 2.2-kb transcript that accumulates in wounded hypocotyls.

In this paper we show that both the mRNA induced by fungal elicitor and the smaller, wound-induced mRNA are transcribed from the HRGP4.1 gene, which encodes an apoprotein with two distinct Pro-rich domains. We have examined the functional properties of the HRGP4.1 promoter by promoter-GUS gene fusion analysis in transgenic tobacco plants. This analysis revealed that 251 bp of promoter sequence upstream of the transcription start site are sufficient for induction by wounding and that this stress response is superimposed on a novel, tissue-specific pattern of promoter activity during normal development, with strong expression in stem nodes and root tips.

MATERIALS AND METHODS

Isolation and Sequencing of the HRGP4.1 Genomic Clone

A bean (*Phaseolus vulgaris* cv Tendergreen) genomic DNA library of partially digested *Sau3A* fragments ligated into λ EMBL4 (Keller et al., 1988) was screened using the 0.7-kb insert from the bean Hyp4.1 cDNA clone as a probe (Corbin et al., 1987). A single clone was isolated and plaque purified. The DNA sequence of the 5' half of the coding region of HRGP4.1 was determined by digestion of the λ genomic clone with *EcoRI* followed by partial digestion with *MnII*. This produced some small overlapping fragments that were cloned into m13mp18 and sequenced by the dideoxy chain-termination method (Sanger et al., 1977).

A 4.1-kb fragment from a *Sau3A* partial digest of the λ clone was subcloned into the *Bam*HI site of pUC19 cm^r, generating plasmid pDC204. A 1-kb *Hae*III fragment from pDC204, containing the 5' flanking portion of HRGP4.1 and a small amount of the coding sequence, was cloned into the *Hinc*II site of pIBI24 (International Biotechnologies,

Inc., New Haven, CT). Two plasmids that contained this insert, one in either orientation, were isolated. A set of nested deletions was generated in the insert of each plasmid using the method of Henikoff (1984), allowing the entire 5' flanking DNA to be sequenced.

A 2.3-kb *Hinc*II fragment from the λ clone that contained all of the coding sequences and a small amount of 3' flanking DNA was subcloned into the *Bam*HI site of pTZ18R (Pharmacia) using *Bam*HI linkers, and the resulting plasmid was called pDC210.2. Oligonucleotides (20-mers) were synthesized (Millipore) for use as primers to determine the sequence of a portion of the 3' coding and noncoding regions of the genomic clone. This plasmid was also used for transposon-facilitated sequencing using the TN1000 sequencing kit from Gold BioTechnology, Inc. (St. Louis, MO), following the manufacturer's instructions. Clones were selected to provide a series of nested insertions in the region of interest. Large plasmid preparations, purified by fast protein liquid chromatography, were prepared for sequencing either manually or on an Applied Biosystems model 373A DNA sequencer using a fluorescent dye-labeled dideoxy terminator kit (Applied Biosystems) and *Taq* polymerase (Perkin-Elmer Cetus). Sequence analysis was performed on a VAX/VMS computer using the University of Wisconsin Genetics Computer Group software package.

Analysis of Transcription Initiation Site

Total cellular RNA (50 μ g) was assayed by primer extension (Boorstein and Craig, 1989) using a 30-mer synthetic oligonucleotide of the sequence: 5'-GTCAGCCAAT-GTTTGGGATGGGAAGAGAGA-3', which primes at positions 75 to 104 relative to the start site of transcription of HRGP4.1.

Promoter-GUS Constructs

Five of the 5' deletions used for sequencing the promoter, containing 914, 657, 459, 251, and 94 bp of 5' flanking sequence, were used for the construction of promoter-GUS constructs. Inserts were removed by digestion with *Hind*III and *Bam*HI and then cloned into similarly digested pBI101.3 (Fig. 1) (Jefferson et al., 1987). To remove the TAG stop codon (between the *Hinc*II and *Bam*HI sites of pIBI24) that was in frame with the translational fusion, each fusion construct was digested with *Bam*HI, filled in with Klenow, cut with *Xba*I, blunt ended with mung bean nuclease, and religated. This removed exactly 6 bp from each, eliminating the stop codon. The sequence of the junction site of the 5' deletion constructs was confirmed by sequencing using a GUS-specific primer.

Constructs containing small internal deletions (pHAE13-MUT1, 2, and 4-7) were generated by a slightly different strategy. Plasmid pDC204 was digested with *Pfl*MI and *Eco*RI, and a 1-kb fragment containing 5' flanking sequence and 75 bp of coding sequence was filled in using the Klenow fragment of DNA polymerase and ligated into pIBI24, which had been digested with *Bam*HI and similarly filled in. This plasmid was then digested with *Hae*III to

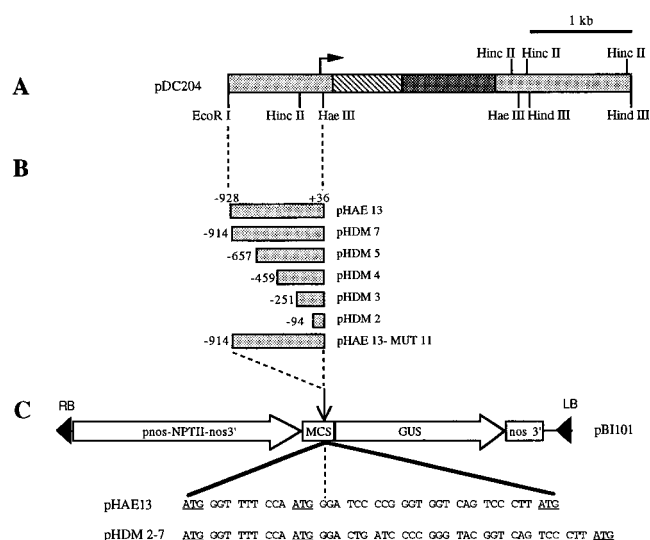


Figure 1. Structure of plasmids containing the HRGP4.1 gene and HRGP-GUS gene fusion. **A**, Partial restriction map of the 4.1-kb bean genomic DNA insert of pDC204. The arrow indicates the site of transcription initiation; the box with diagonal stripes indicates the position of the 16-amino acid repeats; the lightly stippled boxes delineate the 5' (promoter) and 3' flanking sequences; the dark stippled box indicates the position of the 10-amino acid repeats. **B**, Portions of the HRGP4.1 promoter used in seven of the promoter-GUS constructs. **C**, Structure of HRGP4.1 promoter-GUS fusions in the T-DNA of vector pBI101.1. Gene fusion pHAE13 contains 5' flanking sequence from -928 to +36 relative to the transcription initiation site as +1. The dashed vertical line indicates the boundary between the HRGP promoter sequence and pBI101 MCS sequences. The two putative HRGP ATGs and the GUS ATG are indicated in the sequences below the diagram. Gene fusions pHDM-2, -3, -4, -5, and -7 were made by a different strategy from pHAE13 and thus differ at the amino-terminal end of the fusion protein (see "Materials and Methods"). Gene fusion pHAE13-MUT11 has been modified by site-directed mutagenesis to remove 14 bp from the 5' end of pHAE13.

generate a 964-bp fragment extending from 928 bp upstream of the transcription initiation site to 15 bp beyond the putative ATG translation initiation codon (1 bp beyond the second ATG). This fragment was ligated into pBI101.1 (Jefferson et al., 1987), which had been digested with *Bam*HI and filled in. This regenerated the *Bam*HI site and generated a translational fusion. The resulting plasmid, designated pHAE13, was the starting point for site-directed mutagenesis. The junctions between the HRGP4.1 promoter and the GUS gene in pHAE13 and all derivative constructs were confirmed by sequencing.

A *Hind*III/*Bam*HI fragment containing all of the promoter sequences used was cut from pHAE13 and cloned into pBI25, creating plasmid pHYP41.4. A *Hinc*II/*Bam*HI fragment containing promoter elements from -241 to +36 was cut from pHAE13 and cloned into pBI25, creating plasmid pHYP41.3.

Site-directed mutagenesis was performed on either pHYP41.4 or pHYP41.3 by the method of Kunkel (1985), using the Muta-Gene kit (Bio-Rad) to generate small internal deletions spanning the region from -121 to -245. Mutagenic oligonucleotides were synthesized that hybrid-

ized to the template DNA in two places with a gap of 17 to 24 bases in between. The oligonucleotides contained a *Hind*III site at the location of the deletion to facilitate screening of putative mutants. Mutants were characterized first by restriction analysis and then by sequencing. Mutagenesis on plasmid pHYP41.4 was successful only when T4 phage gene 32 protein (Huberman and Kornberg, 1971) was added to the second-strand-synthesis reaction. Following confirmation of each mutagenized sequence, the *Hinc*II/*Bam*HI fragment of pHYP41.3-derived mutants was cloned back into pHYP41.4. Then, the entire mutagenized promoter, contained in an *Xba*I/*Bam*HI fragment, was cloned back into pBI101.1. The deleted nts for each mutant were: pHAE13-MUT1, -246 to -227; pHAE13-MUT2, -230 to -207; pHAE13-MUT4, -214 to -191; pHAE13-MUT5, -195 to -171; pHAE13-MUT6, -170 to -154; pHAE13-MUT7, -150 to -122.

Plant Transformation and Growth

Binary vector constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by a direct freeze-thaw method (Holsters et al., 1978), and leaf discs of tobacco (*Nicotiana tabacum* cv *Xanthi-nc*) were transformed by standard methods (Horsch et al., 1985; Rogers et al., 1986). Transformed plantlets were selected on Murashige-Skoog medium (Murashige and Skoog, 1962) containing 100 μ g/mL kanamycin and 250 μ g/mL carbenicillin. Transgenic plants were clonally propagated in this medium in Magenta boxes (Magenta Corp., Chicago, IL), at 25°C under a 16-h light/8-h dark cycle, until transfer to the greenhouse.

GUS Assays

Root, stem, and leaf tissues were collected from each independent transformant approximately 1 month after transfer to fresh medium, before the stem began to press against the top of the box. Stems were wounded by a procedure similar to one previously used on bean hypocotyls (Showalter et al., 1985). Stem segments, approximately 5 mm in length, were incubated in the dark in 5 mM sodium phosphate, pH 5.5, for 24 h. Tissue for fluorimetric assay was extracted in 1.5-mL microfuge tubes with extraction buffer (Jefferson et al., 1987) and ground glass, using a pestle mounted on an electric drill. GUS activity was determined by the method of Jefferson et al. (1987), and protein was determined by the method of Bradford (1976). Those plants in which extractable GUS activity in all tissues was less than 100 pmol 4-methylumbelliferone $\text{min}^{-1} \text{mg}^{-1}$ protein (about 15% of regenerated plants) were discarded and not included in further analysis.

To determine the time course of wound induction, T_2 progeny of a single transformant, homozygous for the HRGP4.1-GUS gene fusion, were used. Seeds were germinated and grown in the greenhouse until plants were 0.3 to 0.4 m tall. Internode segments were excised from plants between the first and third fully expanded leaves, surface sterilized with 70% ethanol for 30 s, followed by 20% bleach, 0.1% Tween 20 for 15 min, and rinsed three times in

sterile water. Segments were cut into 5-mm-long pieces and then incubated in the dark in 5 mM sodium phosphate, pH 5.5. At various times after excision, segments were removed and frozen in liquid N₂ for later extraction and GUS assay. Segments from four separate plants were assayed at each time. Floral tissues were collected just after anthesis from plants grown in potting soil in a greenhouse.

Histochemical analysis was performed as described by Jefferson et al. (1987). Following fixation in 0.3% formaldehyde, 0.3 M mannitol, and 10 mM 2-Mes, pH 5.6, tissue samples were rinsed three times in 50 mM sodium phosphate, pH 7.0, and vacuum infiltrated with 1 mM X-Gluc (Clontech, Palo Alto, CA) in 50 mM sodium phosphate, pH 7.0. Samples were incubated in this solution at 37°C for 8 h to 3 d as appropriate. Tissue for thin sections was dehydrated in an ethanol series, treated with xylene, embedded in Paraplast (Fisher), and sectioned using a microtome (International Equipment Co., Needham Heights, MA). Paraplast was removed from the sections by two 10-min treatments with xylene. Hand sections were taken from fixed, stained tissue using a double-edged razor blade. Paraffin and hand sections were observed in both the dark-field and bright-field with an Olympus SZH microscope, and micrographs were taken either on Kodak tungsten 50 or on Kodak daylight 100 film using a tungsten illumination filter.

Viral Infection

Transgenic tobacco plants were grown in potting soil in the greenhouse to a height of 30 to 40 cm. Half of the youngest fully expanded leaf was inoculated, in the presence of Carborundum, with 50 µL of 0.01 mg/mL TMV strain U₁ in 20 mM KPO₄, pH 7.8, 1 mM EDTA. The other half of the leaf was mock inoculated with buffer alone. Plants were grown in the greenhouse where supplementary lighting maintained constant daylength; all infections were done between September and November. Lesions were visible 30 to 34 h after inoculation. At 72 h after infection, leaf punches (10–12 per leaf) were taken around individual lesions using a 4-mm-diameter (No. 1) cork borer. Punches were taken randomly from the control half of the leaf. Tissue was either frozen immediately in liquid N₂ and later assayed fluorimetrically for GUS activity or fixed and stained with X-Gluc as described above.

RESULTS

Isolation and Structure of the HRGP4.1 Gene

Labeled insert from the Hyp4.1 cDNA was used to screen a bean (*P. vulgaris* cv Tendergreen) genomic library (Keller et al., 1988). From the genomic clone a 4.1-kb *Sau*3A fragment containing the gene corresponding to Hyp4.1, designated HRGP4.1, was subcloned into pUC19 cm^r, generating the plasmid pDC204. Figure 1A shows a partial restriction map of the pDC204 insert. Attempts to subclone certain fragments from the genomic clone or to generate deletions into the 3' half of the coding region were unsuccessful. The highly repetitive nature of the coding region may cause instability of certain sequences in *Escherichia coli*

(Greener, 1990). Use of a transposon-facilitated sequencing strategy enabled us to sequence recalcitrant portions of the gene.

Approximately 4 kb of pDC204 were sequenced from the *Eco*RI end of the insert. The sequenced fragment contains approximately 1.0 kb of 5' flanking sequence, the entire coding region of the gene, and approximately 1350 bp of 3' noncoding sequence (Figs. 1A and 2). The sequence from 945 to 1635 is collinear with the Hyp4.1 cDNA (Corbin et al., 1987). A putative TATA box is located 29 bp upstream of the transcription initiation site (see section below). Two potential CAAT boxes are found at -159 to -169 (relative to the transcription initiation site as +1). From nts -810 to -767 there is a distinctive motif consisting of TA repeated 22 times. The deduced amino acid sequence indicates the presence of the putative termination codon of the HRGP4.1 protein at nt 2689. There are AATAAA consensus poly(A) addition sequences at nts 2825, 3354, 3529, and 3770.

The deduced protein consists of 580 amino acids divided into two distinct Pro-rich domains. As described previously (Corbin et al., 1987) there is a putative signal peptide of 28 amino acids. This is followed by a Pro-rich domain that contains 14 tandemly repeated copies of the 16-amino acid sequence Ser-(Pro)₄-Lys-His-Ser-(Pro)₄-(Tyr)₃-His. This is followed by a distinctly different motif: 29 repeats of the 10-amino acid sequence Ser-(Pro)₄-Val-Tyr-Lys-Tyr-Lys or variants differing by 1 or 2 amino acids. Within this carboxyl-terminal portion of the molecule a higher order structure can be detected in the amino acid sequence. Every 4 to 7 repeats the 9-amino acid repeat Ser-(Pro)₄-Tyr-Lys-Tyr-Pro appears and is always followed by Ser-(Pro)₅-Tyr-Lys-Tyr-(Ser/Pro) and 2 repeats of Ser-(Pro)₄-Val-Tyr-Lys-Tyr-Lys. The entire protein has a deduced molecular weight of 66,316. Because of the high Lys and Tyr content, the protein is predicted to have a pI of 10.6.

There is a distinct codon usage bias for the amino acid at each position within the repeats. For example, TCT and TCA are always used for the first and second Ser's, respectively, in the 16-amino acid repeat. TAT is the codon used for the first Tyr in 12 of 14 repeats, whereas TAC is used in 13 of 14 repeats at the second and third Tyr. Because of this bias, 37 of the 48 nts encoding the 16-amino acid repeat sequence are invariant, and 4 more differ in only 1 or 2 of the repeats. Similar codon bias is found at many positions in the 10-mer repeats.

Origin of the 2.2-kb and 2.5-kb Hyp4.1 Transcripts

In bean, two transcripts were previously detected by hybridization using the 0.7-kb insert of the Hyp4.1 cDNA as a probe (Corbin et al., 1987). The larger transcript (approximately 2.5 kb) accumulates to high levels in suspension-cultured cells treated with a fungal elicitor and in hypocotyl tissue infected with *Colletotrichum lindemuthianum*, whereas the shorter transcript (approximately 2.2 kb) accumulates in hypocotyls in response to wounding. The shorter transcript was also found in unwounded roots and, to a lesser extent, other tissues (data not shown). The transcription initiation sites for the elicitor- and wound-induced transcripts as well as the transcript present in

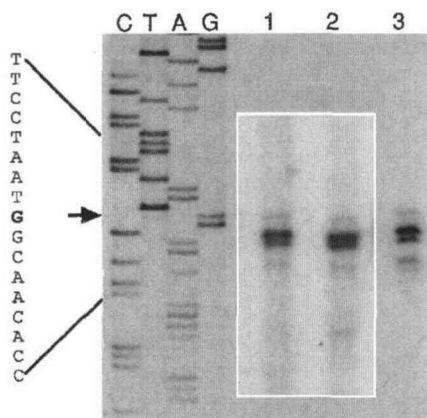


Figure 3. Determination of the transcriptional initiation sites for the different-sized HRGP4.1 mRNAs from different tissues. A 30-mer oligonucleotide priming at positions 76 to 105 relative to the start site of transcription of HRGP4.1 was used in sequencing and in primer extension using 50 μ g of total RNA. The sequence around the start of transcription is indicated on the left, with the presumed start site indicated by an arrow. The sources of RNAs used were as follows: lane 1, roots; lane 2, elicitor-treated suspension-culture cells; lane 3, wounded hypocotyls. A longer exposure is shown for lanes 1 and 2 compared to 3.

were regenerated on medium containing kanamycin and carbenicillin and were analyzed for GUS expression. HRGP4.1-GUS expression was analyzed in 39 independent primary transformants and their progeny.

Tissue-Specific Expression of HRGP 4.1-GUS

Transgenic tobacco plants containing the pHAIE13 promoter-GUS fusion contained approximately 7-fold higher levels of extractable GUS activity in roots compared to stems (Table I). Extractable GUS activity in leaves was at least 10-fold lower than in stems. In 39 independent transformants with detectable GUS activity, there was a wide range in GUS activities (Fig. 4). Those plants with the lowest stem activity had the greatest difference between

stems and roots and between unwounded and wounded stems (Fig. 4, inset). The difference was less pronounced in the plants with the highest basal stem activity. The plants with the highest stem activity (plants Nos. 37, 38, and 39) had GUS activities in stems higher or nearly as high as in roots, the activity was not wound inducible, and activity in leaves was nearly as high as in stems (data not shown). These plants were not included in subsequent calculations of average GUS activities.

Histochemical staining for GUS activity showed that the HRGP4.1 promoter directed a highly specific pattern of expression in different tissues during tobacco development. Figure 5, A through D, shows the pattern of GUS staining in situ at various times during seedling development. The entire seedling was stained 1 d after germination (Fig. 5A), but by 2 d (Fig. 5B) areas of staining in the root tip and hypocotyl were separated by unstained areas. Sometimes cotyledons were stained (Fig. 5C) but usually not (Fig. 5D). In primary transformants grown axenically in Magenta boxes, a high level of GUS activity was seen histochemically in the tips of both primary and lateral roots (Fig. 5, H and I). In the plants with the highest levels of activity, staining was observed in root hairs and, to a lesser extent, other root epidermal cells. Staining was also observed in the cortex of the primary root at the location where a lateral root was being formed but had not yet emerged (data not shown).

Histochemical staining also revealed a high level of GUS activity within stem nodes (Fig. 5, E-G). Sometimes the axillary bud also showed GUS activity. Nodal expression was observed early during seedling development at the junction of the cotyledons (Fig. 5D). In most plants, no GUS activity was detected in internodes by histochemical analysis. In those plants with the highest levels of GUS activity in the stem (>1000 pmol min^{-1} mg^{-1} protein) staining was observed in all stem tissues, with the highest activity in the vascular tissue and the pith (data not shown). GUS activity in floral organs was low in most plants, similar to the level in leaves. One plant, however, showed a highly unusual pattern of expression in stigmas and styles superimposed

Table I. Developmentally induced and abiotic and biotic stress-induced expression of GUS activity in transgenic tobacco containing different HRGP4.1 promoter-GUS constructs

Values represent mean GUS activities derived from duplicate assays of all the transgenic plants containing a particular construct. For stems, roots, and wounded stems, the number of independent plants assayed for each construct is indicated in parentheses to the right of the GUS values for wounded stems. For stigmas, leaf discs, and TMV-infected leaf discs, the number of independent plants assayed for each construct is indicated in parentheses to the right of the GUS values for TMV-infected leaf discs. Four clonally propagated cuttings of pHAIE13 No. 23 were assayed in duplicate. n/d, Not determined. 4-MU, 4-Methylumbelliferone.

Construct	GUS Activity							
	Stem	Root	Wounded stem	Stigma	Leaf	TMV-infected leaf		
			<i>pmol 4-MU min⁻¹ mg⁻¹ protein</i>					
pHAIE13	700	5,216	3,961	71	42	98	(27)	
pHAIE13 No. 23	377	3,547	1,985	12,165	112	2,630	(1)	
pHAIE13-MUT11	1,226	4,567	3,606	20	17	36	(14)	
pHDM-7	106	1,631	1,171	9	4	10	(6)	
pHDM-5	269	1,779	1,339	n/d	14	15	(4)	
pHDM-4	58	609	864	n/d	5	12	(5)	
pHDM-3	67	171	299	n/d	4	3	(2)	
pHDM-2	3	174	6	n/d	11	13	(4)	

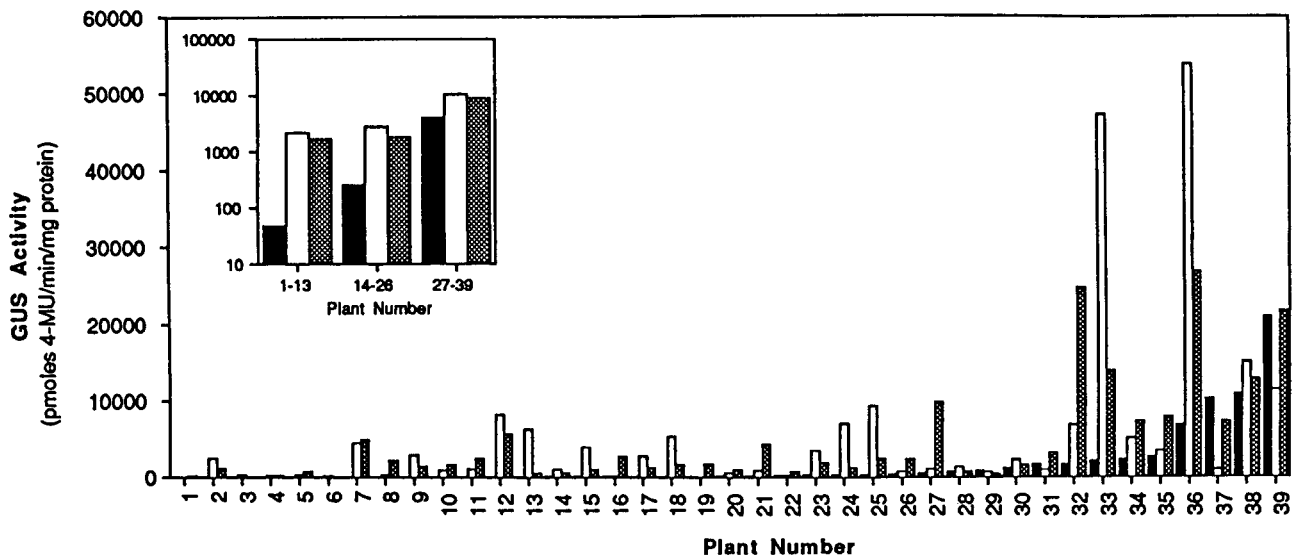


Figure 4. Extractable GUS activities in tissues from pHAIE13 transgenic plants. Stem internode and root tissue were collected from 39 independent primary transformants and values derived from the mean of two replicate assays on each plant. Inset, Mean values for plants with low, medium, and high stem activities (note logarithmic scale of GUS activity). Stems were wounded as described in "Materials and Methods." Plants are ordered by increasing stem activity from left to right. ■, Stem internodes; □, roots; ▨, wounded stems. 4-MU, 4-Methylumbelliferone.

on the pattern typical of the other plants. The specific activity of GUS in stigma extracts of this plant (No. 23) was at least 30 times higher than in the stem and more than 3 times higher than in roots (Table I). Styler expression appeared to be localized to the transmitting tract tissue, and in the stigma the GUS staining was restricted to a band of cells lying just under the epidermis (data not shown). No other flower part in this plant showed unusually high levels of GUS activity.

HRGP4.1-GUS Induction by Wounding and Infection

The kinetics of wound induction of the HRGP4.1-GUS fusion were determined using stem internodes wounded by cutting into small segments (Fig. 6). After a lag of 1.5 h there was a rapid increase in GUS activity. GUS activity doubled within 4 h and increased 6-fold after 48 h. The spatial pattern of wound activation of the HRGP4.1 promoter was examined by histochemical staining for GUS activity *in situ*. Figure 5J shows that wounding leads to the appearance of strong GUS staining in the inner phloem. Staining was also frequently observed in the outer phloem tissue and occasionally in the pith (compare to unwounded stems in Fig. 5, F and G).

Infection by TMV has been shown to induce the production of HRGPs in tobacco (Benhamou et al., 1990). We therefore examined the response of transgenic tobacco containing the HRGP4.1-GUS gene fusion to inoculation with TMV strain U₁. This strain causes the development of necrotic lesions in the *N. tabacum* cv Xanthi-nc genotype that we used. Leaf discs were taken 72 h after infection by TMV from 28 independent transgenic tobacco plants containing the pHAIE13 construct and tested for GUS activity (Table I). Of these plants, 27 showed an average increase of

about 2.3-fold compared to mock-inoculated controls. The plant with anomalous stigma expression (transgenic No. 23) showed a 23-fold average induction (Table I), ranging from 11- to 49-fold in four separate experiments. Histochemical analysis of this plant showed that intense GUS staining was restricted to a ring of tissue surrounding the developing lesion (data not shown).

Functional Analysis of the HRGP 4.1 Promoter

A series of promoter-GUS fusions was constructed in which increasing portions of the HRGP4.1 promoter were deleted from the 5' end (Fig. 1B). In these constructs, the putative translation initiation codon of the HRGP4.1 gene is separated by 3 amino acids from the second HRGP4.1 ATG and by 15 amino acids from the first ATG of the GUS gene, with all 3 ATGs positioned in the same reading frame (Fig. 1C). Transgenic tobacco plants containing these constructs were analyzed for GUS expression in various tissues and compared to plants containing the undelimited promoter-GUS construct (Table I). GUS activities in stems, roots, and wounded stems of plants containing the least-deleted construct, pHDM-7 (-914), were much lower than activities in comparable tissues of plants transformed with pHAIE13 (-928). However, plants containing a construct in which 14 bp had been removed from the 5' end of pHAIE13 (pHAIE13-MUT11) had extractable GUS activities comparable to pHAIE13. This suggests that the difference between the pHAIE13 and pHDM-7 plants was due to the difference in the sequences at the amino-terminal ends of the fusion proteins (Fig. 1C).

There was a decline in activity in both stem internodes and roots as the promoter was successively deleted from -914 to -251 (Table I). Plants containing the -94 pro-

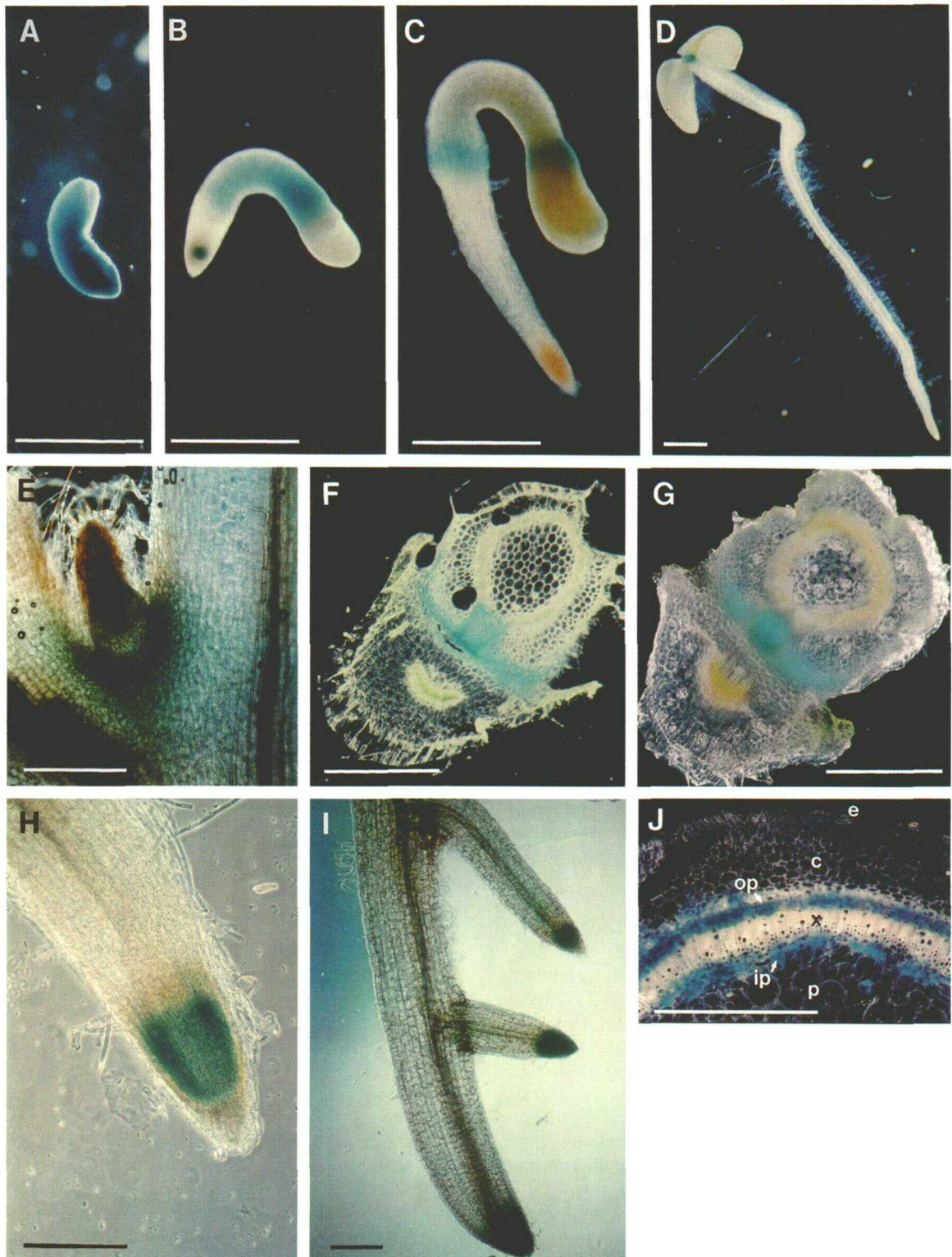


Figure 5. Developmental and tissue-specific expression of HRGP4.1-GUS. A to D, Developing T₂ seedlings (containing the pHAE13 construct) were harvested at various times after germination, fixed, and stained with X-Gluc. The older seedlings had already shed their seed coats (C and D), whereas the younger seedlings were gently squeezed out of their seed coats. Days after germination: A, 1; B, 2; C, 3; D, 4. E to J, Tissue-specific expression of HRGP4.1-GUS in mature plants. E, longitudinal section of an axillary bud and the underlying nodal region; F, lateral section of the nodal region through the axillary bud; G, lateral section of the nodal region directly under the axillary bud; H, close up of a root tip; I, primary and lateral roots; J, lateral section of wounded stem tissue. Five-millimeter stem sections were sliced in half longitudinally, fixed, stained with X-Gluc, and cleared in 70% ethanol. Hand sections were cut across the face of the wounded tissue. c, Cortex; e, epidermis; ip, inner phloem; op, outer phloem; p, pith; x, xylem. Size bars = 1 mm.

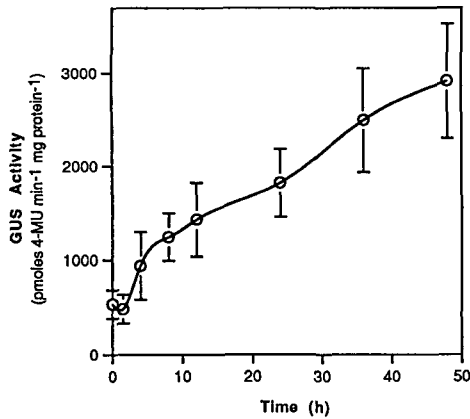


Figure 6. Wound induction of GUS activity in transgenic plants containing the HRGP4.1-GUS gene fusion. Internode segments from T₃ plants containing the pHAE13 construct were excised and incubated for various times prior to extraction and determination of GUS activity. Each point represents the mean activity of segments from four clones of a single transformant and bars denote SD. 4-MU, 4-Methylumbelliferone.

moter-GUS construct (pHDM-2) had GUS activity indistinguishable from that of promoterless control plants. Wound-induced activity also declined, approximately in proportion to the length of the promoter, with no measurable induction in the plants containing pHDM-2. No differences were seen in the distribution of cells expressing GUS in either the nodes or root tips of the deletion construct plants (data not shown).

The effect of the 5' deletions on induction by TMV infection was more difficult to assess. There was an average 2.5-fold induction in six plants containing the pHDM-7 (-914) construct, no induction in the four pHDM-5 (-657) plants, and approximately 2.2-fold induction in the five plants containing the pHDM-4 (-459) construct (Table I). However, only one pHDM-4 plant showed any induction at all, and because it had higher basal levels than the other four plants, it skewed the average. A more definitive analysis of promoter elements controlling induction by infection would require a more sensitive assay and testing a greater number of plants.

To map more accurately promoter elements responsible for the observed pattern of expression, a series of small internal deletions was generated, by site-directed mutagenesis, spanning the region from -251 to -94 in the context of the full promoter (Fig. 7). The effects of most of these small deletions were subtle, resulting in small changes in average GUS activity in whole tissues. For instance, pHAE13-MUT1 and pHAE13-MUT2 were slightly depressed in the level of root activity, whereas pHAE13-MUT2 and pHAE13-MUT4 appeared to be de-repressed in the level of unwounded stem activity. The average values in both stems and roots were lower in plants containing the pHAE13-MUT5 deletion than the unmutated constructs. Transgenics containing pHAE13-MUT6, on the other hand, showed less wound induction on average (approximately 1.6-fold) than those with any other construct.

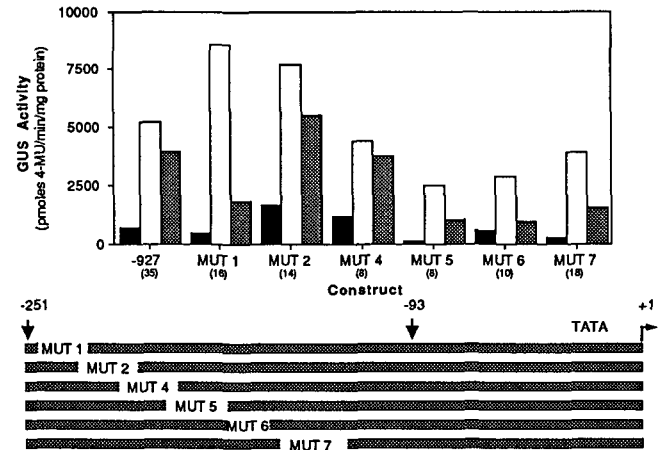


Figure 7. Internal deletion mutants of the HRGP4.1 promoter. GUS activities in extracts of tissue collected from primary transformants containing promoter site-directed deletion constructs. The lower portion of the figure indicates the location of each deletion within the region -251 to +1. The number of independent transformants assayed is indicated beneath each histogram, and the values derive from the means of two replicate assays on all plants containing a particular construct. The amount of sequence deleted varied from 17 to 25 bp. ■, Stem internodes; □, roots; ▨, wounded stems. 4-MU, 4-Methylumbelliferone.

Some internal deletions changed the distribution of GUS activity in root tips as visualized by staining. Between four and nine of the highest expressing plants containing eight different constructs were examined, and the results are summarized in Table II. Roots were scored as positive if they showed any blue color, and most showed the typical pattern shown in Figure 5H. Five of 19 stained roots from pHAE13-MUT2 plants and all of the 11 stained roots from pHAE13-MUT4 plants showed staining only in root hairs or epidermal cells, not in the meristem region as usually seen. No staining was visible at all in any of the roots from

Table II. Presence of root tip GUS staining in transgenic tobacco containing site-directed mutant HRGP4.1 promoter-GUS constructs

Root tips were excised from plants growing in Murashige-Skoog medium and stained for GUS activity as described in "Materials and Methods." The plants tested are those that showed the highest levels of extractable GUS activity in roots. Roots were scored as positive if any visible blue color developed after 24 h of staining.

Construct	Independent Transgenics	No. of Roots	No. of Positives
pHAE13	6	32	25
pHAE13-MUT1	4	22	21
pHAE13-MUT2	4	19	19 ^a
pHAE13-MUT4	4	19	11 ^b
pHAE13-MUT5	4	17	4
pHAE13-MUT6	4	20	0
pHAE13-MUT7	4	17	6
pHAE13-MUT11	9	45	21

^a Five of these roots stained blue only in root hairs and epidermal cells. ^b All 11 roots were blue only in root hairs and epidermal cells.

pHAE13-MUT6 plants, even though roots of these plants all had high levels of extractable GUS activity.

DISCUSSION

HRGP4.1 Gene and Protein Structure

We have isolated and characterized the HRGP4.1 gene, which is a member of a family of genes encoding stress-inducible Tyr-rich HRGPs in bean (Corbin et al., 1987). The corresponding cDNA, Hyp4.1 (Corbin et al., 1987), begins 16 nts downstream from the transcriptional start site and is collinear with the 5' portion of the coding sequence of the HRGP4.1 gene. This region encodes a Pro-rich domain in which the Ser-(Pro)₄ motifs are embedded in a 16-amino acid Tyr-rich unit. The motifs in this sequence are similar to that found in the tomato P3 extensin (Lampert, 1977). In contrast, the 3' region of the HRGP4.1 gene encodes a second Pro-rich sequence in which the Ser-(Pro)₄ units occur in a different higher-order repeat. This second repeat motif resembles, with minor differences, one found in HRGPs from carrot and petunia (Showalter and Rumeau, 1990) and exemplified by the P2 extensin of tomato. A nearly identical repeat sequence was found recently in a partial cDNA clone from soybean (Hong et al., 1994). To our knowledge, the two different repeat sequences found in the HRGP4.1 protein have never before been reported in the same protein, and Kieliszewski and Lampert (1994) hypothesized that these two types of extensin repeats diverged early in evolution. Their convergence on the HRGP4.1 protein may represent a recent recombination or perhaps the first example of a retained ancestral form.

Two transcripts, one of 2.2 kb isolated from wounded hypocotyls and unwounded roots and one of 2.5 kb isolated from elicited cell cultures, initiate at the same site 20 nts upstream from the putative translation initiation codon. The two transcripts may arise from differential splicing of a putative intron in the 3' untranslated region of the gene.

Based on the hydroxylation pattern of known extensin proteins, it is likely that only the 12 isolated Pro's will remain unhydroxylated in the mature protein, whereas all of the Pro's in Pro₄ blocks (95.5%) are expected to be hydroxylated and thus highly arabinosylated (Kieliszewski et al., 1995). The presence of a different Tyr-rich higher-order repeat sequence at each end of the apoprotein is likely to have a major impact on the extent and pattern of oxidative cross-linking of the mature glycoprotein (Kieliszewski and Lampert, 1994). This may be an important factor in determining the final functional architecture of the walls of cells in which the HRGP4.1 gene is expressed.

The distinctive (TA)₂₂ repeat found in the HRGP4.1 flanking DNA is not unique to this gene. A search of the data bases revealed no fewer than 25 sequences containing (TA)_n stretches where *n* ranged from 21 to 37. The repeat is found in the 5' flanking region of several stress-inducible and developmentally regulated plant genes including patatin, PAL, soybean lectin, and glycinin (Cramer et al., 1989; Rocha-Sosa et al., 1989; Kitamura et al., 1990; Lindstrom et al., 1990). It is also found in the introns or 3' flanking regions of several plant genes. Our deletion analysis

showed no apparent functional role for this sequence, nor did deletion analysis of the promoters of glycinin A or bean PAL (Leyva et al., 1992; Itoh et al., 1993).

Stress Activation

Transgenic tobacco plants containing an HRGP4.1-GUS gene fusion were used to study the functional properties of the HRGP4.1 promoter. Our analysis showed a strong wound induction from low basal levels of activity in tobacco stem internodes. Marked increases in GUS activity were observed within 4 h after wounding, and the kinetics of promoter activation by wounding inferred from the time course of the appearance of GUS activity are consistent with previous measurements of the accumulation of HRGP4.1 mRNA in wounded bean hypocotyls (Showalter et al., 1985; Corbin et al., 1987; Sauer et al., 1990). The parallel histochemical assay of GUS activity in situ showed that rapid wound activation of the HRGP4.1 promoter was localized primarily to the outer phloem with somewhat weaker expression in the inner phloem. Reinforcement of cell walls in the outer phloem by accumulation of HRGP4.1 may be important in preventing entry of pathogens into the vascular system at wound sites. This hypothesis is consistent with the observation of wound induction in phloem parenchyma of a gene encoding PAL, an enzyme involved in the synthesis of phytoalexin antibiotics and lignin precursors (Liang et al., 1989; Leyva et al., 1992).

It was surprising that the HRGP4.1 promoter was not as highly activated by infection with TMV as by wounding, since a marked increase in HRGP4.1 transcript levels following infection was previously reported (Showalter et al., 1985; Corbin et al., 1987). However, this is consistent with the operation of different intercellular stress signals in response to wounding and microbial attack, inferred from the differential accumulation of two different-sized transcripts from the HRGP4.1 gene in wounded bean hypocotyls and hypocotyls infected with *C. lindemuthianum* (Corbin et al., 1987). In an incompatible interaction between bean and *C. lindemuthianum*, the expression of the HRGP4.1 mRNA was 10- to 20-fold higher in the tissue immediately adjacent to the hypersensitively responding cells than in more distant tissue (Showalter et al., 1985). In situ hybridization showed that this reflected accumulation of HRGP4.1 transcripts in the epidermis and in the outer layers of the vascular region (Templeton et al., 1990). We may not have included enough 5' flanking DNA in our promoter-GUS constructs to confer a normal pattern of gene expression in transgenic tobacco. Promoter elements controlling infection inducibility of the HRGP4.1 gene may be located further upstream or perhaps in 3' flanking regions. The much higher levels of induction by infection found in transgenic No. 23 suggests that elements outside the introduced transgene can dramatically enhance the response to pathogen infection without influencing wound inducibility. It is also possible that the appropriate promoter elements for infection induction are present but that aspects of the signal transduction pathways in bean responding to *C. lindemuthianum* and tobacco responding to TMV differ enough that full activation does not occur.

Tissue-Specific Developmental Expression

The stress-induced activation of the HRGP4.1 promoter is superimposed on a novel, tissue-specific developmental pattern of expression. Although promoter-reporter analysis with the GUS transgene can be artifact prone, especially in heterologous systems (Oommen et al., 1994), our results define a clear pattern of developmental expression consistent with the proposed functions of Hyp-rich proteins. Expression of the HRGP4.1-GUS gene fusion was detected histochemically in root tips and to a lesser extent along the rest of the root, in a distinct region of the node and sometimes in the axillary bud. Expression in nodes was initiated early in development, shown by GUS staining between the cotyledons and in the nodes associated with primordia for the first true leaves. However, no expression was detected in the shoot apical meristem itself. Activation of the HRGP4.1 promoter in the node and root tip suggests a role for the HRGP4.1 protein in the strengthening of the cell walls of these tissues, where the mechanical properties of the cell wall may be particularly important. Thus, root tips experience compressional forces as they push their way through the soil, and the node undergoes both compressional and tensional forces as it supports the petiole and its leaf. Deposition of the Tyr-rich HRGP4.1, with a high capacity for oxidative cross-linking, could potentially make a contribution to cell wall reinforcement in these specific tissues. HRGP accumulation has been observed in root tips of maize and soybean (Ludevid et al., 1990; Ye and Varner, 1991).

Nonspecific enhancement or repression of gene expression has been noted by a variety of workers and is known as the position effect. It is generally assumed that the position of a transgene may affect its relative expression level, but not its pattern of expression, in promoter analysis in transgenic plants. This assumption may not always be valid in view of the demonstration of downstream regulatory elements determining tissue-specific expression of some plant genes (Dietrich et al., 1992; Larkin et al., 1993). In the present study, we found that 3 of 39 plants containing the -928 to +35 HRGP4.1 promoter-GUS construct did not show the same distribution of expression as the rest; expression was dramatically enhanced in stems and leaves. Another plant showed extremely high (and quite specific) expression in stigmas and styles. This same plant showed a markedly enhanced activation by TMV infection. Thus, some highly specific promoter elements are presumably able to act at a distance (that is, from somewhere outside the T-DNA) and influence the expression of the HRGP4.1 promoter.

Functional Promoter Analysis

Analysis of promoter 5' deletion constructs showed that the region from -251 to +36 was sufficient to give the same developmental expression pattern as the full-length promoter, albeit at a reduced level. The expression pattern was lost when the promoter was deleted to -94 bp. This suggests that sequences between -928 and -251 serve mainly to enhance expression, whereas sequences between -251 and -94 control the basic pattern. None of the small deletions made in the -251 to -94 region was successful in

eliminating the activity of the promoter, suggesting that there may be some redundancy of promoter elements. Even deletion of a sequence from -170 to -154 (MUT6) reduced only the average level of wound induction from 5.7- to 1.6-fold but did not completely eliminate it. This same deletion eliminated all histochemically detectable GUS activity in the root tip. It may be that an endogenous stress signal released in wounding is also present at the stem/petiole junction and in growing root tips. We do not know the nature of that signal, but we have seen no induction of GUS expression in our transgenic plants by either ethylene or salicylic acid (K.L. Wycoff and R.A. Dixon, unpublished results).

The complex tissue-specific expression of the HRGP4.1 promoter is clearly distinct from that of the HRGPnt3 promoter (Keller and Lamb, 1989). These observations support the hypothesis that selective expression of specific cell wall structural protein genes is an important mechanism in determining the functional architecture of cell walls during cell differentiation. The HRGPnt3 gene is expressed exclusively in a subset of pericycle and endodermal cells involved in lateral root initiation and is not induced in response to environmental stress. In contrast, the HRGP4.1 promoter shows a much less limited pattern of expression, with activation by wounding and pathogen attack, superimposed on a distinctive pattern of developmental expression in root tips and stem nodes. These data suggest that selective deposition of the Tyr-rich HRGP4.1 may be deployed as a general mechanism for increasing wall rigidity and strength in mechanically stressed tissues and tissues vulnerable to, or under, pathogen attack. Since HRGP4.1 is very Tyr rich compared to other developmentally expressed HRGPs (Keller and Lamb, 1989; Sauer et al., 1990; Wycoff et al., 1992), we propose that HRGP4.1 expression may be a mechanism to increase the capacity for oxidative cross-linking in cell walls.

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