Developmental and Environmental Regulation of a Bean Chalcone Synthase Promoter in Transgenic Tobacco

Jürg Schmid,1 Peter W. Doerner,2 Steven D. Clouse,3 Richard A. Dixon,2 and Christopher J. Lamb2,3

Plant Biology Laboratory, Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037

Plant Biology Division, Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, Oklahoma 73402

Regulatory properties of a 1.4-kilobase promoter fragment of the bean chalcone synthase CHS8 gene were examined by analysis of glucuronidase (GUS) activity in transgenic tobacco containing a CHS8-GUS gene fusion. The promoter was highly active in the root apical meristem and in petals, exclusively in those cells of the inner epidermis that accumulate anthocyanins. The gene fusion was only weakly expressed in other floral organs, mature leaves, and stems. The early stages of seedling development were characterized by an apparent wound induction of the promoter in the endosperm and strong expression in the immature root, which became localized to the apical meristem and perivascular tissue at the root-hypocotyl junction. The promoter became active during lateral root formation in both the new root and damaged tissue of the main root. The gene fusion was also expressed in greening cotyledons and primary leaves but not in the shoot apical meristem. Light modulated expression in the cotyledons and root-shoot junction but had no effect on other aspects of the developmental program. Wounding or fungal elicitor treatment of mature leaves activated the promoter in a well-defined zone adjacent to the stress site. Stress induction occurred in mesophyll and vascular tissues as well as in the epidermis. We conclude that the CHS8 promoter contains cis-elements required to establish temporal and spatial control of flavonoid biosynthesis during development and in response to diverse environmental stimuli.

INTRODUCTION

Plants respond flexibly to environmental stimuli that act upon a plastic program of development. A key feature of many of these responses is the synthesis of flavonoid natural products, which have diverse functions in development and interactions with the environment. Thus, flavonoids are important for the pigmentation of flowers and seed coats and in protection against UV irradiation (Hahlbrock and Scheel, 1989). In legumes, pterocarpans and isoflavonoids derived from flavonoid precursors function as phytoalexins (Dixon et al., 1983), and simple flavonoids such as naringenin and luteolin are rhizosphere signals for the induction of nod genes in Rhizobium (Long, 1989). Moreover, recent data indicate that certain flavonoids inhibit the binding of napthylphthamic acid to its cellular receptor and, hence, may have regulatory functions as modulators of polar auxin transport (Jacobs and Rubery, 1988).

Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetyl units from malonyl-CoA with 4-hydroxycinnamoyl-CoA to give naringenin chalcone, which is the first committed step in the branch pathway of phenylpropanoid metabolism specific for flavonoid biosynthesis. CHS mRNA and enzyme levels are highly regulated during development associated with the tissue- and cell type-specific accumulation of flavonoid pigments and in response to environmental stimuli for the synthesis of flavonoids involved in adaptation or protection (Hahlbrock and Scheel, 1989; Lamb et al., 1989). Thus, CHS is a key metabolic control point and provides an excellent system for analysis of the molecular mechanisms governing natural product biosynthesis. Run-on transcription assays in isolated nuclei and transient expression of CHS-reporter gene fusions in electroporated protoplasts have shown that UV irradiation of parsley cell cultures and elicitor treatment of bean cell cultures stimulate CHS transcription to initiate the synthesis of UV protectants and phytoalexins, respectively (Lawton and Lamb, 1987; Dron et al., 1988; Lipphardt et al., 1988; Schulze-Lefert et al., 1989). Functional assays of the activities of mutated promoters in electroporated protoplasts have identified the region of a bean CHS promoter required for elicitor induction (Dron et al., 1988) and defined cis-acting elements involved in UV in-
duction of parsley and antirrhinum CHS promoters (Liphardt et al., 1988; Schulze-Lefert et al., 1989).

In contrast, relatively little is known about the transcriptional properties of CHS promoters in intact plants. Run-on transcription assays indicate that wounding and fungal infection stimulate CHS transcription in bean hypocotyls (Lawton and Lamb, 1987) and an antirrhinum CHS promoter-chloramphenicol acetyltransferase gene fusion is light regulated in transgenic tobacco (Kaulen et al., 1986). We are interested in how these responses to environmental stimuli are integrated at the gene level within the developmental program of natural product biosynthesis. The regulation of CHS genes from legumes is of particular interest in this regard because of the additional biological functions of flavonoid derivatives in legumes as phytoalexins and nod gene inducers superimposed upon their ubiquitous functions as pigments and UV protectants. In this paper we have examined the regulatory properties of the promoter of the bean CHS8 gene by analysis of β-glucuronidase (GUS) activity in transgenic tobacco plants containing a CHS8-GUS gene fusion. CHS8 is one of seven CHS genes in the bean genome and was selected for the present study because it is a highly expressed member of the gene family (Ryder et al., 1987). Our data demonstrate that the CHS8 promoter contains the cis-acting elements required to establish genetic control of tissue- and cell type-specific biosynthesis of flavonoids during development and also to respond to diverse environmental stimuli.

RESULTS

Organ-Specific Expression of Bean CHS Genes

Poly(A)+ RNA isolated from leaves, stems, roots, flowers, and floral buds of greenhouse-grown bean plants was subjected to RNA gel blot analysis. The coding sequence of the CHS15 gene (Ryder et al., 1987) was used as nick-translated probe with hybridization and washing conditions that did not distinguish between different CHS transcripts. Figure 1 shows that the highest levels of CHS transcripts were found in roots, several times less in stems, and even less in leaves, mature flowers, and floral buds. The contribution of CHS8 transcripts to overall CHS expression in different organs was determined by S1 nuclease protection analysis. In previous studies induction of CHS8 transcripts was observed in elicitor-treated cell suspension cultures and in wounded hypocotyls (Ryder et al., 1987). Figure 2 shows that there was a very strong accumulation of CHS8 transcripts in roots. Low levels of the CHS8 transcript were present in stems and floral buds, whereas transcript levels in fully expanded leaves and mature flowers were too low to be detected. Shorter protected fragments corresponding to other CHS transcripts showed distinctly different patterns of accumulation, indicating differential regulation of CHS8 transcripts compared with transcripts of other members of the gene family. For example, the major CHS transcript in floral buds corresponded to the CHS4 cDNA.

Transformation of Tobacco with CHS8 Constructs

The CHS8 promoter containing 1250 bp upstream of the transcription start site and 162 bp of the untranslated leader sequence extending to 1 bp 5′ of the translation initiation site (Figure 3A) was fused to the GUS coding sequence in the vector pBI101.1 (Figure 3C) (Jefferson et al., 1987). In addition, the complete CHS8 gene was ligated into the polylinker of the binary vector BIN19 (Figure 3B) (Bevan, 1984). Both constructs were transferred to to-
Figure 2. S1 Nuclease Protection Analysis.

Poly(A)+ RNA isolated from different bean tissues and in vitro transcribed control RNA were subjected to S1 nuclease protection analysis to determine the contribution of CHS8 transcripts to the overall accumulation of CHS transcripts. Control "sense" RNA was generated with T7 RNA polymerase after linearization of plasmids pCHS1 3' 25, pCHS4 3' 25, pCHS4a 3' 25, pCHS14 3' 25, and pCHS17 3' 25 (Figure 3E) with EcoRI. The probe for S1 nuclease protection analysis was made by excision of a 475-bp BspMI-PvuII fragment (Figure 3E) from pCHS14 3' 25 and filling in the end with radiolabeled nucleotide triphosphates. Genomic clone CHS8 corresponds to cDNA clone CHS14 (Ryder et al., 1987).

(A) Labeled probe (lane 1), 123-bp ladder (lane 2), and probe hybridized without added RNA and subjected to S1 digestion (lane 3).

(B) Probe hybridized with the following sense RNAs before S1 nuclease digestion: CHS1 (lane 4), CHS4 (lane 5), CHS4a (lane 6), CHS14 (lane 7), and CHS17 (lane 8). The lengths of protected probe are as expected from CHS cDNA sequence data (Ryder et al., 1987).

(C) Probe hybridized with 2.5 μg each of poly(A)+ RNA from leaves (lane 9), stems (lane 10), roots (lane 11), flowers (lane 12), floral buds (lane 13), and total RNA (6 μg) from wound-induced bean hypocotyls (lane 14). The 370-bp fragment protected by the probe corresponding to the CHS8 transcript is marked by the arrow. (C) was exposed 50 times longer than (B).

Figure 3. Restriction Maps of Genomic Clone CHS8 and Constructs Used for Tobacco Transformation and Molecular Analysis.

(A) Partial restriction map of genomic clone CHS8. The 3.9-kb EcoRI-HindIII fragment contains 1.4 kb of 5'-untranslated sequences followed by a coding region of 1270 bp and a 3'-untranslated region of 1.2 kb. A single intron (boxed) of 103 bp is located toward the 5' end of the coding sequence.

(B) The 3.9-kb EcoRI-HindIII fragment subcloned into BIn19.

(C) The chimeric CHS8-GUS gene construct comprising the 5'-untranslated sequences from the EcoRI site up to a Ddel site 1 bp upstream of the initiation codon ligated in front of the GUS coding region (GUS) followed by a nos terminator (NOS-T) in the vector pBl101.1.

(D) The same promoter fragment ligated into Smal-restricted pSP64.

(E) CHS cDNA clone pCHS14 (Ryder et al., 1987) restricted at the conserved Stul site and at the EcoRI site created during cDNA synthesis and ligated into Smal-EcoRI-cut pBl225. Corresponding constructs were made for pCHS1, pCHS4, pCHS4a, and pCHS17 (Ryder et al., 1987). Scale bar applies to (A) through (D), (E) is 2 times this scale. The arrows above the gene indicate the transcription start site.
Figure 4. RNase Protection Analysis.
Total RNA (50 μg) isolated from the roots of axenically grown transgenic tobacco plants and from wounded bean hypocotyls was subjected to RNase protection analysis: probe not digested with RNase (lane 1); RNA from two independent transformants carrying the entire CHS8 gene (Figure 3B): CHS8-4 (lane 2) and CHS8-13 (lane 3); RNA from tobacco transformed with vector BIN19 (lane 4); RNA (6 μg) from wounded bean hypocotyls (lane 5); RNA from roots of tobacco transformant CHS8-GUS-8 carrying the CHS8-GUS gene fusion construct shown in Figure 3C (lane 6); tRNA control reaction (lane 7); end-labeled 123-bp ladder as size markers (lane M).

Organ-Specific Expression of the CHS8-GUS Gene Fusion
The expression of the CHS8-GUS gene fusion in different organs of the transformants CHS8-GUS-5, CHS8-GUS-7, and CHS8-GUS-8 was monitored by fluorimetric assay of extractable GUS activity (Table 1). In vegetative organs there was a high level of GUS activity in roots, moderate activity in stems, and low activity in extracts of mature leaves. In floral organs only very low levels of extractable GUS activity were observed with sepals, pistils, and stamens. High levels of GUS activity were found in petals, confined to the small apical regions that accumulate anthocyanins. There was only very low GUS activity in the remaining nonpigmented regions, which comprise the major portion of the petal in tobacco.

Although the same overall pattern of expression of CHS8-GUS was observed in each plant examined, there was, as expected, some quantitative variation among these independent transformants. In particular, transformat CHS8-GUS-7 exhibited less marked expression of the gene fusion in stems and in the pigmented regions of petals compared with transformants CHS8-GUS-5 and CHS8-GUS-8. However, the level of expression in roots of CHS8-GUS-7 was comparable with that observed in the other transformants.

Spatial Pattern of CHS8-GUS Expression
The spatial pattern of CHS8 promoter activity underlying the strong expression of the CHS8-GUS gene fusion in roots and petals was examined by histochemical analysis of GUS activity in situ using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-glucuronide. Figure 5A confirms that the CHS8-GUS gene fusion was specifically expressed in the pigmented regions of petals. There was an abrupt transition from tissue showing strong histochemical staining for GUS to tissue showing no appreciable GUS activity. The limits of CHS8 promoter activity corresponded precisely with the boundary between the pigmented and nonpigmented regions. To examine the spatial pattern of CHS8 promoter activity at the cell level, petals were fixed after completion of the GUS chromogenic reaction and then embedded in paraffin for preparation of thin sections. Figure 5B shows that GUS staining was confined to cells in the inner epidermis with no significant staining of the outer epidermis or internal vascular and parenchymal tissues. Moreover, at the junction between pigmented and nonpigmented tissues there was an abrupt transition zone of only a few epidermal cells between those showing GUS staining and those showing no GUS staining (Figure 5C). This junction corresponded exactly to the boundary between pigmented epidermal cells, which show a central conic projection, and the adjacent nonpigmented cells, which lack this morphological feature in tobacco.

Figure 5D shows that GUS activity could also be histochemically detected in pollen grains in situ. Only about half the pollen grains exhibited GUS activity, consistent with segregation analysis of the gene fusion in progeny from the selfed primary transformant CHS8-GUS-8. The CHS8-GUS gene fusion did not appear to be expressed in the tapetal layer of the anther surrounding the pollen sac. No GUS activity was detected in the pollen grains from trans-
Table 1. Organ Distribution of GUS Activity in Transformants Containing the CHS8-GUS Gene Fusion

<table>
<thead>
<tr>
<th>Organ</th>
<th>CHS8-GUS-5</th>
<th>CHS8-GUS-7</th>
<th>CHS8-GUS-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>22</td>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>Stems</td>
<td>315</td>
<td>70</td>
<td>150</td>
</tr>
<tr>
<td>Roots</td>
<td>6000</td>
<td>6900</td>
<td>7200</td>
</tr>
<tr>
<td>Sepals</td>
<td>2</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Pistils</td>
<td>7</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Stamens</td>
<td>7</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Petals (pigmented tissue)</td>
<td>6000</td>
<td>480</td>
<td>8400</td>
</tr>
<tr>
<td>Petals (nonpigmented tissue)</td>
<td>5</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

genic plants transformed with the promoterless GUS plasmid pBl01.1 (data not shown).

Figures 5E to 5J show that the CHS8 promoter was active at the apical tip of the mature main root. The GUS activity was confined to a specific region of the root tip. Thus, no GUS activity was observed in the root cap, the root epidermis, or the root vascular system. Strong expression of the chimeric construct was observed in the apical meristem and in the immediately adjacent zone of cell elongation. Expression of the gene fusion progressively weakened distal to the apical tip, becoming restricted to a group of cells cylindrically surrounding the developing vascular system. No significant GUS activity was detectable histochemically in more mature tissues behind the root tips. A similar spatial pattern of GUS activity was observed in the tips of mature lateral roots.

Developmental Regulation

The histochemical analysis of GUS activity in roots and petals indicated that the CHS8 promoter exhibited well-defined spatial patterns of activity in these organs. To trace the initial developmental program of CHS8 gene expression, we monitored promoter activity during germination and the early stages of seedling development by histochemical analysis of GUS activity in the progeny of selfed transformant CHS8-GUS-8. Figure 6A shows that the CHS8 promoter was activated in the endosperm of germinating seeds, apparently as a wound response at the site of radicle penetration and emergence. This response was observed in all seed tested and did not segregate in the new seedlings, indicating that this represented induction of the gene fusion in cells possessing the maternal genotype.

In contrast, expression of GUS activity in developing seedlings segregated with the chimeric construct and represented regulation of the promoter in the genomes of the progeny. In the germinating seedling a low level of GUS activity was observed diffusely throughout the cotyledon and radicle (Figure 6B) until the emergence of the latter, when strong expression of the gene fusion was seen in the immature root (Figure 6B). Initially, expression was observed throughout the root from the tip region to the root-hypocotyl junction, but as the cotyledons began to green and expand, GUS staining in the root became restricted to the apical tip, in the characteristic tissue-specific pattern observed in mature roots (see above), and to the region of the root-hypocotyl junction (Figure 6C). GUS activity at this junction was predominantly localized to a group of cells cylindrically enveloping the vascular bundle and was absent from the epidermis (Figure 6D). GUS staining at the root-hypocotyl junction progressively diminished and no activity was detectable at the developmental stage where the cotyledons were fully expanded (Figure 6E). In contrast, the root tip continued to show strong staining throughout the remaining course of seedling development and in the adult plant.

Figures 5K to 5N show that the CHS8 promoter was also activated in lateral root development. Localized GUS staining could be observed at an early stage (Figure 5K) as the new root was initiated from within the pericycle of the main root. During penetration through the cortex and epidermis of the main root, strong GUS staining was observed throughout the lateral root, and the surrounding damaged tissues of the main root also stained for GUS activity (Figures 5L to 5M). After emergence of the lateral root, GUS activity was confined to the tip of the new root in a pattern similar to that in the tip of the main root (Figure 5N).

The CHS8 promoter also became active in cotyledons during the expansion and greening of these organs (Figure 6F). When the cotyledons were fully expanded, GUS staining was observed in blocks across the cotyledon not correlated with any morphological feature. The strength of staining increased over time as the cotyledons matured. Sectioned cotyledons showed CHS8 promoter activity throughout epidermal and parenchymal tissues within these patches (Figure 6G). Very strong GUS staining was also observed in the primary leaves as they emerged (Figure 6F). No GUS activity was histochemically detectable within the apical meristem, which was in contrast to
Figure 5. Histochemical Localization of GUS Activity in Floral Organs and Root Tissues of Transgenic Tobacco Plant CHS8-GUS-8.
transgenic tobacco seedlings transformed with PAL2-GUS constructs (Liang et al., 1989), which show GUS activity within the tip of the apical meristem and also in the developing vascular system (Figure 6H).

**Light Interactions with the Developmental Program**

Light had a marked effect on some aspects of this developmental program but little effect on other aspects. Thus, in the cotyledons of 10-day-old etiolated seedlings, the CHS8 promoter was active only at the extreme tip and did not become active throughout the rest of the organ in the absence of a light signal (Figure 6K). If these etiolated seedlings were exposed to white light for 13 hr, the spatial pattern of GUS activity rapidly changed to give blocks of staining across the greening cotyledon, characteristic of the pattern observed in seedlings germinated and grown under a 16-hr light/8-hr dark cycle (Figure 6L). In developing roots the progressive localization of CHS8 promoter activity at the apical tip did not appear to be light dependent, occurring in etiolated seedlings as in normal light-grown seedlings. However, the decay in promoter activity at the root-hypocotyl junction was delayed in etiolated seedlings (Figure 6I). Exposure of etiolated seedlings to white light for 4 hr greatly accelerated the disappearance of GUS staining in this region, which was complete after 13-hr illumination (Figure 6J).

**Stress Activation**

The induction of the CHS8-GUS gene fusion in seed tissues during germination and in tissues of the main root during emergence of a lateral root indicate that the CHS8 promoter was activated by tissue damage incurred during normal development. Figure 7 shows that the CHS8 promoter was also responsive to externally imposed stress. Mature leaf tissue of transgenic plants containing the CHS8-GUS gene fusion contained very low levels of GUS activity as monitored by fluorimetric assay in leaf extracts (Table 1). Localized wounding of the tissue of a detached mature leaf caused induction of GUS activity in the rings of tissue immediately adjacent to the wound sites. While strong GUS staining was observed at all wound sites after 48 hr to 72 hr (Figure 7A), only weak staining at some sites was observed 16 hr after wounding. However, immediate application of fungal elicitor to the wound site caused a marked induction of the CHS8 promoter after 16 hr to 24 hr compared with the response to wounding alone (Figure 7D). Strong GUS staining was observed in tissue immediately adjacent to the site of elicitor application (within 2 mm to 3 mm) and weaker staining in tissue up to 10 mm to 15 mm distant. Activation of the CHS8 promoter at a distance was also observed in transgenic tobacco leaves infiltrated locally with an incompatible isolate of Pseudomonas syringae or treated with potassium oxalate (B. Stermer, J. Schmid, C.J. Lamb, and R.A. Dixon, unpublished observations).

To examine tissue and cell type specificity of stress induction of the CHS8 promoter, leaf fragments containing a wound site were embedded and sectioned after the histochemical GUS reaction. These sections revealed inducible CHS8 promoter activity in a sharply delimited region surrounding the lesion (Figures 7B and 7C). Induction of GUS activity was observed in leaf epidermal, mesophyll, and vascular tissue within this zone.

**DISCUSSION**

Transgenic plants containing the CHS8-GUS gene fusion exhibited specific and characteristic patterns of GUS activity during plant development and in response to environmental stimuli. Although we cannot completely rule out...
Figure 6. Histochemical Analysis of CHS8-GUS Expression during Seedling Development.
some bias in the observed patterns of GUS activity at the cell level due to differential uptake and availability of the chromogenic substrate, the patterns we observed are very different from those observed in transgenic plants containing cauliflower mosaic virus 35S-GUS gene fusions or a number of other plant promoter-GUS gene fusions including PAL2-GUS (Benfey and Chua, 1989; Bevan et al., 1989; Liang et al., 1989). Hence, the patterns of GUS activity in the CHS8-GUS transgenic plants are established by the specific properties of the CHS8 promoter.

The bean promoter is appropriately regulated in transgenic tobacco. Thus, the gene fusion was transcribed from the same start site as that utilized by endogenous bean CHS genes in wounded bean hypocotyls and by the entire CHS8 gene in transgenic tobacco. Moreover, the pattern of CHS8 promoter activity in transgenic tobacco, as inferred from the induction of GUS activity, closely resembled the activity of the endogenous promoter in bean, as monitored by the accumulation of CHS8 transcripts. Thus, the high levels of GUS activity in tobacco roots and the induction of the gene fusion by wounding and fungal elicitor closely paralleled the pattern of accumulation of CHS8 transcripts in bean.

Likewise, anthocyanin pigments accumulated at a much earlier stage of floral development in bean compared with tobacco, and, hence, the high levels of GUS activity in the pigmented regions of petals of transgenic tobacco could be correlated with the presence of CHS8 transcripts in bean floral buds even though the transcripts were no longer detectable in mature petals. The very high levels of GUS activity in the pigmented regions of mature petals of the transgenic tobacco plants, compared with the modest level of the CHS8 transcript in bean floral buds, may reflect prolonged translation of the GUS mRNA throughout petal development.
development after expression of the gene fusion at an early stage in flowering. Moreover, RNA was extracted from the whole floral bud. GUS activity was low in floral organs other than petals, and within petals, high GUS activity was restricted to the pigmented tissue such that the specific activity of GUS in extracts of nonpigmented petal tissue was at least 30-fold lower than in extracts exclusively from the pigmented tissue. Expression of the CHS8-GUS gene fusion exclusively in the cells of the inner epidermis that accumulate anthocyanins provides further evidence that the CHS8 promoter is appropriately regulated in tobacco flowers.

Overall, the present data indicate that the transcriptional activity of the CHS8 promoter is a major factor in determining the pattern of accumulation of the corresponding transcript in intact plants during development and in response to light, wounding, or fungal elicitor. The close correlation between expression of the CHS8-GUS gene fusion and accumulation of flavonoid natural products is consistent with the hypothesis that selective transcription of the CHS8 gene is a major control site in flavonoid synthesis both during development and in response to environmental stimuli. Examples include, in addition to expression in petals associated with anthocyanin synthesis, strong expression in roots correlated with the synthesis of nod gene inducers and other root flavonoids, as well as stress induction associated with the synthesis of isoflavonoid phytoalexins (Wiermann, 1981; Dixon et al., 1983). Interestingly, nodules form in the cortical tissues adjacent to the vascular cylinder rather than in more peripheral tissues (Long, 1989). Hence, the tissue-specific expression of CHS8 in roots may be important in establishing the site of nodule development by localized synthesis of nod gene inducers.

A surprising feature of the CHS8 promoter is the strong expression in the apical meristem of the root and at sites of lateral root initiation. However, the CHS8 promoter is not active in all meristems because no GUS staining was observed at the shoot apex, whereas the bean PAL2 promoter is active in this meristem (Figure 6H and Liang et al., 1989). Because certain flavonoids may modulate polar auxin transport, CHS8 expression in root meristems could be involved in determining the distribution of auxin and, hence, modulation of root morphogenesis. Analysis of the effects of exogenous auxin and auxin-insensitive mutants suggests that auxin plays a critical role in lateral root initiation (Wightman et al., 1980). At these sites, the CHS8-GUS gene fusion appears to be activated not only in cells of the new root but also in surrounding tissues of the main root. This is unlikely to reflect spreading of the GUS stain because GUS staining exclusively in cells of the lateral root tip as it penetrates the tissues of the main root was observed in transgenic tobacco plants containing the hydroxyproline-rich glycoprotein HRGPn3 gene promoter-GUS gene fusion (Keller and Lamb, 1989). Induction of CHS8 in the damaged tissue of the main root may represent a defense mechanism against microbial infection at the site of emergence of the lateral root.

Tobacco phytoalexins are furanocoumarin and sesquiterpenoid compounds rather than flavonoids, and endogenous CHS genes are not stress induced (Dixon et al., 1983). However, the bean CHS8 promoter is activated by wounding or elicitor in transgenic tobacco even though CHS has no function in stress metabolism in tobacco. Therefore, the CHS8 promoter responds to a conserved signal system for stress induction of defense genes that was established before the evolution of chemically distinct phytoalexins in legumes and solanaceous plants. Deposition of lignin is a ubiquitous defense response, and induction of genes encoding phenylpropanoid biosynthetic enzymes involved in the synthesis of lignin monomers, e.g., PAL, 4-coumarate:CoA ligase, and cinnamyl alcohol dehydrogenase, by wounding, fungal elicitor, or infection has been observed in a wide range of monocot and dicot species (Dixon and Harrison, 1990). The evolution of flavonoid derivatives as phytoalexins in legumes may, thus, have been accompanied by the incorporation into the CHS8 promoter of cis-elements conserved in the PAL promoters of many species. It will be of interest to determine whether genes encoding enzymes of the phenylpropanoid branch pathway for coumarin synthesis from plants where furanocoumarins function as phytoalexins, e.g., parsley (Dixon et al., 1983), are stress induced when transferred into legumes.

The distinct zone of induction of the CHS8 promoter by wounding or fungal elicitor is reminiscent of the spatial pattern of accumulation of defense gene transcripts in response to infection of parsley leaves with Phytophthora megasperma (Schmelzer et al., 1989). Such a pattern, which our data indicate is established at the level of gene transcription, cannot readily be accounted for by a single diffusible signal because this would be expected to give a gradation between strongly induced cells and noninduced cells. Hence, induction of the CHS8 promoter may involve two or more signals with different transmission properties or a signal that is transmitted by local serial progression, cell to cell. It is striking that within the response zone, the CHS8 promoter is stress activated in all tissues throughout the cross-section of the leaf. This is in marked contrast to the tissue- and cell type-specific expression observed in many facets of the developmental program of regulation. Hence, the promoter contains cis-acting elements responsive to externally applied signals that override or circumvent regulatory elements that establish the strict tissue and cell type specificity exhibited in other contexts. Activation in all tissues adjacent to a local wound may be crucial for effective protection against microbial attack after mechanical damage.

The expression of the CHS8-GUS gene fusion resembles the pattern of activation of the bean PAL2 promoter in transgenic tobacco with respect to wound induction and strong expression in roots and the pigmented regions of
petals (Bevan et al., 1989; Liang et al., 1989). Moreover, the PAL2-GUS and CHS8-GUS gene fusions exhibit the same complex program of expression in the early stages of seedling development with GUS staining initially delocalized throughout the root and then becoming restricted to the root tip and the root-hypocotyl junction (S. Ohl and C.J. Lamb, unpublished observations). However, in stems the PAL2 and CHS8 promoters exhibit markedly different patterns of expression. Thus, the CHS8 promoter is only weakly expressed in stems compared with roots, whereas the PAL2 promoter is active in stems at high levels comparable with those in roots. This reflects strong PAL2 expression in differentiating xylem associated with lignin synthesis in which CHS is not involved. Moreover, whereas both promoters are active in root apical meristems, only the PAL2 promoter is active in the shoot apical meristem.

Some common sequence motifs in CHS and PAL promoters have recently been discerned that function in the induction of transcription in suspension cultured cells by UV irradiation and elicitors (Cramer et al., 1989; Lois et al., 1989; Lawton et al., 1990). Experiments to determine the functions of these and other elements in the regulation of CHS and PAL genes in transgenic plants are in progress. Moreover, these studies should reveal the functional architecture of these promoters by which exquisite tissue- and cell type-specific developmental regulation is combined with flexible responses to diverse environmental stimuli. The complex regulation of the CHS8 promoter demonstrated in the present study provides an experimental basis for examination of the molecular mechanisms underlying key aspects of the unique developmental plasticity of higher plants.

METHODS

CHS8-GUS Gene Fusion and Probe Construction

The complete CHS8 gene on a 3.9-kb EcoRI-HindIII fragment of the bean genomic clone CHS-A8 was ligated into the polylinker of the BIN19 binary vector (Bevan, 1984) as shown in Figure 3. A 1.4-kb EcoRI-Ddel fragment of this clone containing 1250 bp 5' of the transcription start site and 162 bp of the untranslated leader sequence to 1 bp upstream of the translation initiation codon was ligated into the SmaI site of the promoterless GUS expression vector pBI101.1 (Jefferson et al., 1987) after filling in the ends (Figure 3C). The same fragment was also ligated into the Smal site of plasmid pSP64 (Figure 3D) (Promega Biotec). Both constructs were verified by restriction site analysis and sequencing (Sambrook et al., 1989) over the new junction with GUS-specific (Clonetech) or SP6-specific primers, respectively. Reagents for S1 nuclease protection analysis were made by restricting CHS cDNA clones pCHS1, pCHS4, pCHS4a, pCHS14, and pCHS17 (Ryder et al., 1987) at the conserved Stul site and at the EcoRI site created during cDNA synthesis. These fragments were ligated into Smal-EcoRI-cut pBl25 (IBI), to give the plasmids pCHS1 3' 25, pCHS4 3' 25, pCHS4a 3' 25, pCHS14 3' 25, and pCHS17 3' 25, respectively.

Plant Transformation and Growth

The vectors containing the CHS8 gene or the CHS8-GUS gene fusion were transferred from Escherichia coli HB101 into Agrobacterium tumefaciens LB4040 by a triparental mating with E. coli containing the mobilization plasmid pRK2013 (Ditta et al., 1980). Tobacco leaf disc transformation and regeneration of transgenic plants were performed as described in Rogers et al. (1986). Primary transformants were grown at 25°C under a 16-hr light (115 μE)/8-hr dark cycle.

RNA Analysis

Total cellular RNA was isolated from bean tissues, wound-induced hypocotyls, and tobacco roots as described in Bell et al. (1986) and further purified over a CsCl gradient (Sambrook et al., 1989). Poly(A)* RNA was isolated by oligo(dT)-cellulose affinity chromatography (Sambrook et al., 1989) and used for RNA gel blot and S1 nuclease protection analysis. For RNA gel blot analysis, RNA samples and an RNA ladder (Bethesda Research Laboratories) were electrophoresed through a 1.2% formaldehyde agarose gel, transferred to nitrocellulose, and processed further as described in Sambrook et al. (1989). Prehybridization and hybridization were performed under standard conditions (Sambrook et al., 1989); the final wash of filters was at 60°C in 0.3 × SSC, 0.5% SDS (1 × SSC = 150 mM NaCl, 15 mM Na citrate, pH 6.5). Autoradiography was performed with Kodak RP film and intensifier screen at −70°C.

S1 nuclease protection analysis was performed as described by Sambrook et al. (1989). "Sense" RNA was generated with T7 RNA polymerase after linearization of plasmids pCHS1 3' 25, pCHS4 3' 25, pCHS4a 3' 25, pCHS14 3' 25, and pCHS17 3' 25 with EcoRI. The probe for S1 nuclease protection analysis was made by excising a BspM1-PvuII fragment (Figure 3E) from pCHS14 3' 25 and filling in the end with radiolabeled nucleotide triphosphates. RNase protection was performed as in Sambrook et al. (1989). The template DNA for the "antisense" RNA probe (see Figure 3D) was linearized with EcoRI before in vitro transcription with SP6 RNA polymerase.

CHS8-GUS Expression

Developmental and environmental regulation of the CHS8 promoter were studied in seedlings germinated from surface-sterilized seeds of selfed primary transformant CHS8-GUS-8. After imbibition in H2O, seeds were kept at 4°C overnight, and further germination was carried out in basal MS medium (Linsmaier and Skoog, 1965) without glucose (Sigma) under the same light conditions as primary transformants or in complete darkness. Leaves of primary transformants were wounded either by gently crushing leaves with flat forceps or by pricking with an Eppendorf tip to give a 2-mm to 3-mm hole and treating the wound site immediately with 5 μL of sterile distilled H2O or 5 μL of fungal elicitor (100 μg of glucose equivalent/ml). The elicitor was the high molecular weight fraction heat released from washed mycelial walls of P. megasperma f. sp. glycinea (Ayers et al., 1976).
GUS Assays

GUS activity was assayed in tissue extracts by fluorimetric determination of the production of 4-methylumbelliferone from the corresponding glucuronide (Jefferson, 1987). Protein was determined by the method of Bradford (1976), and GUS activity was expressed as picomoles of product per minute per milligram of protein. Histochemical localization of GUS activity in situ was performed by incubation of plant tissues at 37°C in 50 mM sodium phosphate (pH 7.0) containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (1 mM) after fixation in 0.3% formaldehyde as described by Jefferson (1987). Tissues for embedding were subsequently dehydrated through solutions of increasing ethanol concentration. After thorough dehydration in 100% ethanol, samples were cleared in four changes of xylene, two changes of paraffin-saturated xylene, and four changes of molten paraffin. Embedded samples were then sectioned to 10-μm thickness. Ribbons were floated onto glass slides, dried, deparaffinized, and mounted with acrylic resin.

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