The Plant Cell, Vol. 5, 241–251, March 1993 © 1993 American Society of Plant Physiologists

RESEARCH ARTICLE

Identification of a Methyl Jasmonate–Responsive Domain in the Soybean *vspB* Promoter

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Soybean *vspB* encodes a highly expressed vegetative storage protein-acid phosphatase. In soybean, *vspB* expression is stimulated by methyl jasmonate (MeJA) and sugars. The *vspB* promoter was studied by transforming tobacco with fusions of 5′ noncoding *vspB* DNA and the gene encoding β-glucuronidase (GUS). Constructs containing 833 bp of *vspB* 5′ DNA showed high expression of GUS in stems, leaf veins and trichomes, sepals, and pollen. Sucrose (0.2 M) and MeJA (10^{-5} M) increased gene expression when applied to leaf tissue. Deletion of the region −787 to −520 with respect to the transcription initiation site rendered the *vspB* promoter noninducible by MeJA but still sucrose responsive. This result indicates that DNA elements capable of modulating *vspB* by MeJA can be separated from carbon response elements. Further 5′ end deletion from −520 to −403 or 3′ end deletion from −165 to −289 removed DNA sequences involved in carbon modulation of gene expression. A DNA domain that mediates the MeJA response was further localized to a 50-bp region between −535 and −585. This domain when fused to a cauliflower mosaic virus (CaMV) 35S truncated (−88) promoter makes the CaMV promoter responsive to MeJA. The MeJA-responsive domain contains a G-box motif (CACGTG) and a C-rich sequence. A similar 50-bp DNA region is present in the putative promoter of *vspA*. Related sequences are located in a wound- and MeJA-responsive domain of the proteinase inhibitor II gene and a UV-responsive promoter domain of *chs*, the gene encoding chalcone synthase that is also responsive to MeJA.

INTRODUCTION

The soybean vegetative storage proteins VSPα and VSPβ are vacuolar glycoproteins that are abundant in apical tissues of soybean seedlings, including stems, cotyledons, and young leaves (Mason et al., 1988; Staswick, 1989a; Mason and Mullet, 1990). In more mature plants, the VSPs and their mRNAs accumulate in developing leaves, stems, pods, and flowers but only to low levels in roots, mature seeds, and mature leaves (Staswick, 1989a; Mason and Mullet, 1990). The VSPs accumulate in leaves before anthesis, decline during pod filling, and increase again after seed maturation (Wittenbach, 1982; Staswick, 1989a). Removal of developing pods from soybean plants or petiole girdling to block transport from leaves causes the accumulation of the VSPs and their mRNAs (Wittenbach, 1982; Staswick, 1989b). In fact, if pods are continually removed, the VSPs can constitute up to 45% of the soluble leaf protein (Wittenbach, 1983). This pattern of expression is consistent with the role of the VSPs as a temporary sink for carbon and nitrogen in the shoot, as proposed by Wittenbach (1983).

VSPα and VSPβ are 80% identical in amino acid sequence (Mason et al., 1988; Staswick, 1988) and exist as homo- and heterodimers (Spilatro and Anderson, 1989). They are localized in the vacuoles of paraveinal mesophyll and bundle sheath cells of leaves (Franceschi and Giaquinta, 1983) and at lower levels in the leaf epidermis (Staswick, 1990). We have recently shown that the VSPs are acid phosphatases with some specificity for polyphosphates (DeWald et al., 1992). The VSPs are encoded by the genes *vspA* and *vspB*; analysis of genomic clones indicates that the *vsp* genes comprise a small family containing at least three genes (Rapp et al., 1990; Rhee and Staswick, 1992a, 1992b).

Several lines of evidence indicate that jasmonic acid (JA) or methyl jasmonate (MeJA) is a primary regulator of *vsp* expression (Mason and Mullet, 1990; Anderson, 1991; Franceschi and Grimes, 1991; Staswick et al., 1991). Elevated expression of the *vsp* in soybean seedling hypocotyl hooks and lower expression in roots is paralleled by higher concentrations of MeJA in hooks and lower levels in roots (Mason et al., 1992). *vsp* mRNA accumulation in wounded soybean tissue is preceded by an increase in JA/MeJA in the wounded tissue (Creelman et al., 1992). Inhibitors of lipoxygenase that have the potential to inhibit JA/MeJA biosynthesis also block *vsp* mRNA accumulation in wounded tissue (Staswick et al., 1991). Furthermore, the *vsp* genes are inducible in leaves and cell cultures by JA/MeJA at 10^{-8} to 10^{-5} M (Anderson, 1991; Mason and

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Mullet, 1990; Staswick, 1990). Induction of *vsp* mRNA in response to wounding (Mason and Mullet, 1990; Staswick, 1990) suggests that these proteins may have some function in plant defense, in addition to their role in substrate storage. Other wound-inducible, MeJA-sensitive genes encode chalcone synthase (*chs*), proline-rich protein-2 (Creelman et al., 1992), and proteinase inhibitors (Farmer and Ryan, 1990).

We have shown that sugars (sucrose, glucose, and fructose) and MeJA act synergistically to induce the accumulation of *vsp* mRNAs in soybean leaves and cell cultures (Mason et al., 1992). Consistent with this finding, the wound response in leaves is greatly enhanced by light and inhibited by treatment with the photosynthetic electron transport inhibitor DCMU (Mason et al., 1992). A similar requirement for light or sucrose is observed during the wound induction of proteinase inhibitor I (PI-I) in tomato (Green and Ryan, 1973) and inhibitor II (PI-II) in potato (Johnson and Ryan, 1990), and the induction of potato *pin2*, which encodes proteinase inhibitor IIK, is greatly enhanced by sucrose (Palm et al., 1990; Kim et al., 1991). These data indicate that wound induction of some plant genes may require both metabolic and hormonal signals for optimal expression.

In this study, we show that the *vspB* promoter is responsive to sugars, MeJA, and wounding in transgenic tobacco plants. Deletion analysis showed that DNA sequences mediating jasmonate and sucrose responses can be separated. In addition, a 50-bp DNA region was found to confer MeJA responsiveness when fused to a cauliflower mosaic virus (CaMV) 35S truncated (−88) promoter.

RESULTS

vspB 5′ End Confers Responsiveness to MeJA, Sucrose, and Wounding

We isolated an XbaI-PstI soybean genomic fragment that contains the region −1451 to +82 with respect to the transcription initiation site of *vspB* (Rhee and Staswick, 1992b). This fragment was truncated at +46, 7 bp upstream of the *vspB* initiation codon, and fused to the coding region of the β-glucuronidase (*gus*) gene. A DNA fragment from the 3′ end of *vspB* (+1768 to +2344) was obtained by polymerase chain reaction (PCR) of the parent genomic clone and fused to the 3′ end of the *vspB* promoter–*gus* fragment to yield construct XV, as shown in Figure 1. Substitution of the nopaline synthase (*nos*) terminator in place of the *vspB* 3′ end gave construct X (Figure 1).

The *vspB-gus* fusions were used to transform tobacco plants. Nine independent transformants containing the XV construct and two containing the X construct were rooted in soil. At 6 and 8 weeks of age, they were tested to determine whether *gus* expression was responsive to MeJA. A 5-mM suspension of MeJA (5 μL) or water (next lower leaf) was applied to the adaxial side of adjacent leaves, and after 24 hr, 1-cm-square leaf sections were excised and GUS activity was determined.

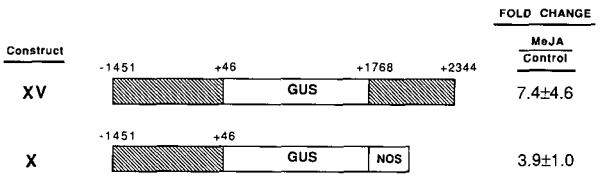

Figure 1. *vspB* 5′ DNA Mediates Response to MeJA in Transgenic Tobacco.

gus fusions containing the *vspB* 5′ region (−1451 to +46) and either the *vspB* 3′ region (+1768 to +2344, construct XV) or the *nos* 3′ region (construct X) were used to transform tobacco. Transformants were tested for MeJA-induced *gus* expression by the spotting assay described in the text. Fold change indicates the ratio of mean ± SD GUS activity in MeJA-treated leaves to that in water-treated leaves for two or more independent transformants for each construct. Segments of the *vspB* 5′ and 3′ noncoding DNA are represented by hatched boxes. The mean basal activities expressed as picomoles per minute per milligram of protein are 103.2 ± 53.7 for construct XV and 33.5 ± 29.9 for construct X.

Figure 1 shows that MeJA increased GUS expression 7.4-fold in the XV plants and somewhat less (3.9-fold) in the X transformants. These data indicate that the *vspB* 5′ domain alone conferred responsiveness to MeJA.

Expression of *vspB* in soybean plants is modulated by MeJA, wounding, and sugars (sucrose, glucose, and fructose) (Mason et al., 1992). To extend the analysis described above, a second *vsp* promoter–*gus* construct was prepared that contained the *vspB* 5′ end domain −787 to +46 fused to *gus* (H constructs). The influence of sucrose and MeJA was assayed using excised leaves. Figure 2 shows that incubation of excised leaves in the light with cut petioles in water caused a slight stimulation of GUS activity over unexcised controls. The increase in expression in excised leaves may be caused by small amounts of JA produced as a consequence of excision (Creelman et al., 1992). GUS activity was increased two- to threefold in excised leaves by the uptake of 0.2 M sucrose, and somewhat less by uptake of 10 μM MeJA. Treatment with both sucrose and MeJA yielded an additive stimulation of GUS activity. Wounding of leaves still attached to transgenic plants by crushing the distal one-third of the leaf several times with a hemostat also caused an increase in GUS activity. Transgenic plants containing the X and H constructs (five independent transformants) responded similarly to these treatments.

To further analyze induction by sucrose/MeJA, single mature leaves from two independent H transformants were assayed for GUS activity after treatment with different media. To avoid possible leaf position effects, each leaf was cut into pieces and pieces were randomly distributed among different media. Midrib and lateral veins were excluded from the samples because higher basal GUS activity was associated with these tissues. As shown in Figure 3A, excised controls that were incubated in the light showed approximately a doubling of GUS activity. Increased expression in these samples was probably due to wound-induced JA accumulation. Sucrose or

glucose provided at 0.2 M induced a tripling of activity in the dark, whereas mannitol caused no increase. MeJA at 10 μM induced a 2.5-fold increase in GUS activity in the light but not in the dark, which suggests a requirement for photosynthetic assimilate, as previously observed in soybean (Mason et al., 1992). The highest GUS activity was obtained by culturing the leaf pieces with sucrose and MeJA together, which induced a fivefold increase over the unexcised control. In contrast, tobacco plants transformed with the CaMV 35S promoter fused to *gus* showed no response to sucrose or MeJA either in the dark or the light, except for a slight decrease in activity with MeJA in the light or in the presence of sucrose (Figure 3B).

The *vspB* Promoter Responds to Physiological Levels of Sucrose and MeJA

To determine the optimal levels of sucrose and MeJA for *vspB* promoter activity, we characterized the dose responses of two different *vspB-gus* constructs to sucrose and MeJA. Figure 4 shows the changes in GUS activity for transformants X1 (−1451, Figure 4A) and H2 (−787, Figure 4B) at different sucrose concentrations in the excised leaf section assay. Both constructs showed a maximal stimulation at 200 mM sucrose when no exogenous MeJA was present. When MeJA was supplied at 1 μM, further stimulation was observed. Interestingly, the stimulating effect of 1 μM MeJA was greater for the X construct (Figure 4A) than for the H construct (Figure 4B).

The responses of *vspB-gus* transformants X1 and H2 to different levels of MeJA are shown in Figure 5. The sucrose

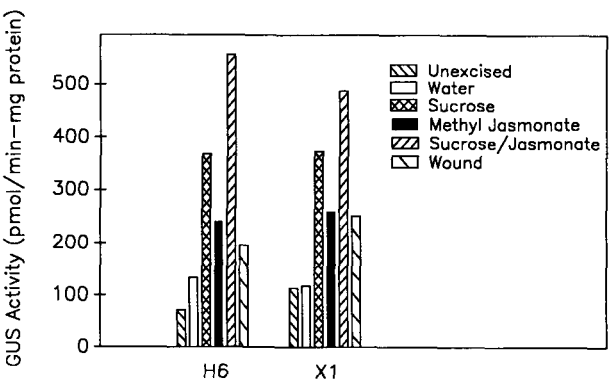

Figure 2. The *vspB* Promoter Is Responsive to Sucrose and MeJA in Transgenic Tobacco.

R_0 transformants H6 (−787 to +46) and X1 (−1451 to +46) were grown in soil until each had six fully expanded leaves. One leaf was sampled for the unexcised control and others were excised at the petiole/node junction, cut again under water, and their cut petioles placed in water, 0.2 M sucrose, 10 μM MeJA, or 0.2 M sucrose/10 μM MeJA, and incubated in the light for 24 hr. Another leaf was wounded with a hemostat on the distal one-third of the lamina, left on the plant, and incubated in the light for 24 hr. The basal one-third of each leaf, excluding the midrib, was harvested, extracted, and assayed for GUS activity.

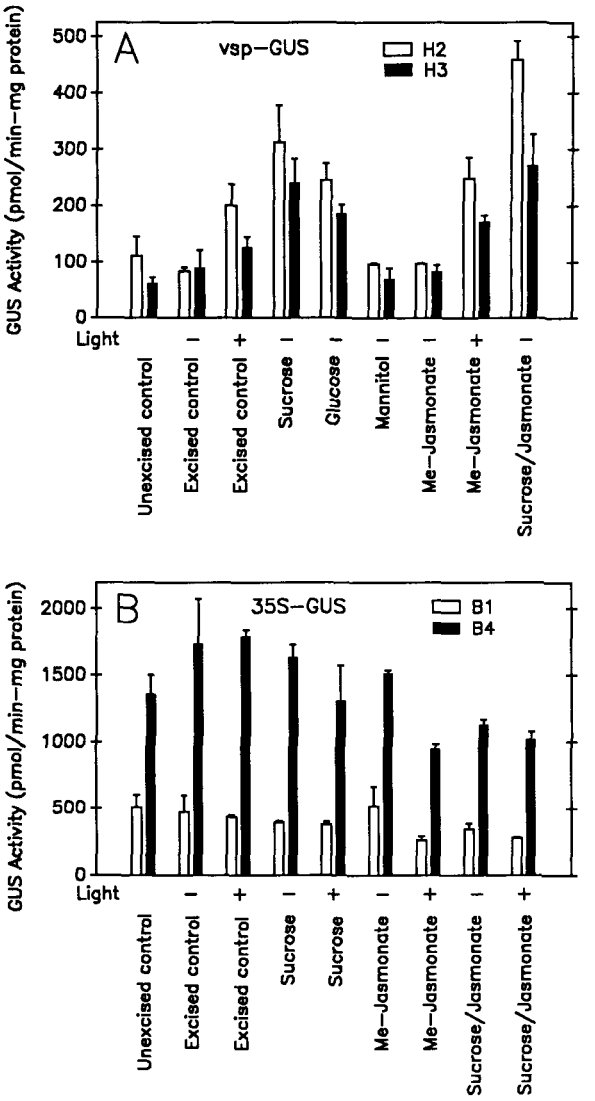

Figure 3. The *vspB* Promoter Is Responsive to Sugars, MeJA, and Wounding in Cut Leaf Pieces From Transgenic Tobacco.

(A) Independent R_0 transformants H2 and H3 (containing the sequence −787 to +46) were grown in soil until several leaves were fully expanded. A single fully expanded leaf was excised from each plant, surface sterilized, and cut into interveinal pieces ~1.0 cm^2. Three leaf pieces were harvested at random for the "unexcised control." Other pieces were floated on solutions of Murashige and Skoog salts plus vitamins (excised control) containing 0.2 M sucrose, 0.2 M glucose, 0.2 M mannitol, 10 μM MeJA, or 0.2 M sucrose/10 μM MeJA, and incubated in the light (+) or dark (−), as indicated for 24 hr. Leaf pieces were extracted and assayed for GUS activity. Data are the means ± SD of values obtained from three different leaf pieces.

(B) Tobacco plants were transformed with pBI121 containing the CaMV 35S promoter fused to the *gus* coding region. Independent R_0 transformants B1 and B4 were grown in soil and leaf pieces treated as in **(A)**. Data are the means ± SD of values obtained from three different leaf pieces.

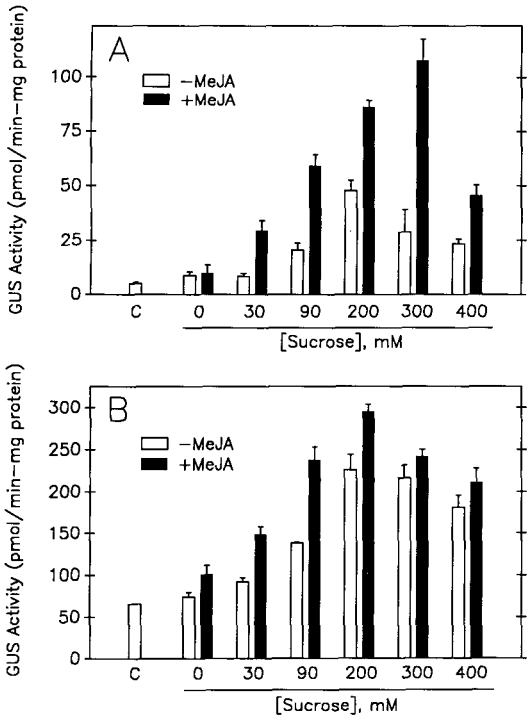

Figure 4. Sucrose Dose Response of *vspB* Promoter Fragments in Transgenic Tobacco.

(A) R_1 transformant X1 (−1451 to +46) was grown from seed and mature interveinal leaf pieces were obtained, as given in Figure 3. Three pieces were harvested as untreated controls (C) and others were floated on solutions containing Murashige and Skoog salts, vitamins, and different amounts of sucrose ± 1 μM MeJA for 24 hr in the dark. Treatments with (+MeJA) or without (−MeJA) MeJA are indicated. GUS activities are the means ± SD of values obtained for three different leaf pieces. **(B)** Data obtained are as given in **(A)** for R_1 transformant H2 (−787 to +46).

concentration in these assays was 90 mM, which induced a substantial increase in GUS activity over the unexcised control level without the addition of exogenous MeJA (Figure 5, treated versus untreated controls). Both constructs showed a stimulation over the treated control level by addition of MeJA at 10^{-8} M and were maximally stimulated at $\sim 10^{-6}$ M MeJA. The enhancement of GUS activity by MeJA over the sucrose-treated control level was again greater for the X construct than for the H construct. For example, at 1 μM MeJA, the X1 transformant showed a fourfold stimulation over the treatment with sucrose alone and a 16-fold stimulation over the unexcised control, whereas the H2 transformant showed only two- and fivefold enhancements, respectively.

Localization of *vspB-gus* Expression in Transgenic Tobacco Tissues

The distribution of GUS activity in leaves, petioles, and stem internodes of two independent *vspB-gus* transformants containing the H construct (−787 to +46) is shown in Figure 6. The highest activity with both transformants was seen in petioles and internodes, whereas leaf tissue had lower activity. Histochemical staining for GUS activity in transformant H2 is shown in Figure 7. GUS activity was localized largely in stems, petioles, and veins of untreated plants; little staining was observed in roots. In the stems, staining was especially heavy at the nodes around the base of the axillary buds (Figures 7A and 7C) and at the apex just below the apical bud (Figure 7B). In leaves, GUS activity was most abundant in the petioles (Figure 7E). The staining in petioles and veins was associated with the bicollateral phloem and the parenchyma mainly on the adaxial side (Figure 7E). The lamina appeared free of GUS activity except for trichomes, which stained heavily (Figure 7D). Flowers showed GUS activity localized in the pedicel and receptacle (Figures 7F and 7G), the style just below the stigma (Figure 7G), the sepals (Figure 7H), and pollen (Figure 7I).

Deletions of the *vspB* Promoter Alter Its Response to Sucrose and MeJA

To begin delineation of sequences that are important in sucrose and MeJA responses, we generated several deletions of the 5′ and 3′ ends of the *vspB* promoter and fused them to the *gus* coding region, as shown in Figure 8. Transformants were screened for responses to sucrose and MeJA by the excised leaf section assay and by spotting MeJA directly on leaves. As shown in Figures 2 to 5, *vspB-gus* constructs that have 5′ ends at position −1451 or −787 yielded transformants

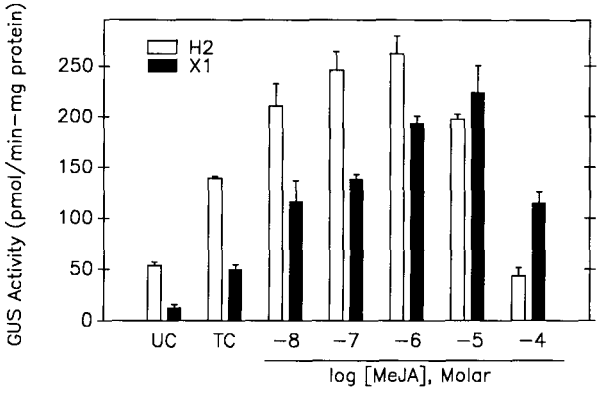

Figure 5. MeJA Dose Response of *vspB* Promoter Fragments in Transgenic Tobacco.

R_1 transformants of X1 (−1451 to +46, see Figure 2) and H2 (−787 to +46, see Figure 3) were grown from seed and mature interveinal leaf pieces were obtained, as given in Figure 3. Three pieces were harvested for untreated controls (UC) and others were floated on solutions containing Murashige and Skoog salts, vitamins, 90 mM sucrose, and different amounts of MeJA for 24 hr in the dark. Treated controls (TC) were floated on the solutions given above with no MeJA. GUS activities are the means ± SD of values obtained for three different leaf pieces.

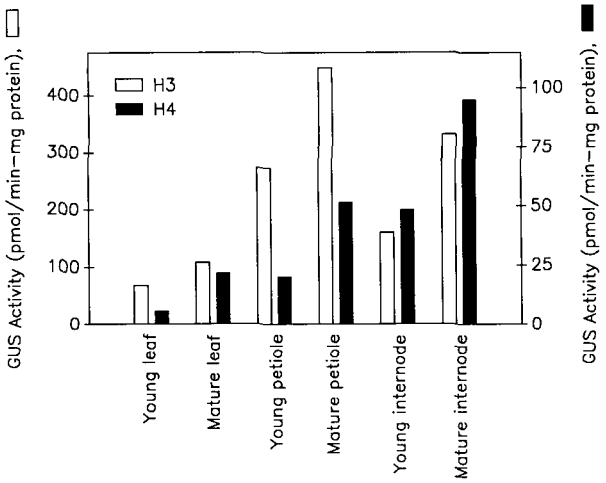

Figure 6. Distribution of GUS Activity in Organs of *vspB-gus* Transgenic Tobacco.

Tobacco plants were transformed with *vspB* promoter fragments H (−787 to +46) fused to the *gus* coding sequence in pBI101. Independent R_0 transformants H4 and H3 were grown in soil to the stage of incipient flowering and tissues were sampled, extracted, and assayed for GUS activity.

that were responsive to both sucrose and MeJA. Figure 8 shows that deletion of the 5′ end of the *vspB* promoter to position −520 removed enhancement of GUS activity by MeJA. In fact, inclusion of 10 μM MeJA in the medium with 0.2 M sucrose inhibited the induction observed with sucrose alone. Further deletion to position −403 largely removed the sucrose response, but a slight MeJA response was observed. Interestingly, when the 5′ end was deleted to position −277, some sucrose enhancement was regained. Constructs with 5′ ends truncated at position −206 showed little response to either sucrose or MeJA. The 5′ −51 to +46 fragment of the *vspB* promoter, when fused to *gus*, showed only a basal level of GUS activity and no sucrose or MeJA inducibility in six independent transformants.

The 3′ deletions were fused to the blunted BglII site at −51, which served as a minimal promoter. The 3′ deletions to positions −165, −289, and −407 yielded transformants that retained MeJA inducibility, although the deletion from −289 to −407 reduced the response. This result suggests that sequence elements in the −289 to −407 domain interact with the sequence elements/factors upstream of position −520 in mediating the response to MeJA. Transformants harboring 3′ deletions to position −165 showed enhanced GUS activity in response to sucrose treatment, but plants transformed with 3′ deletions to position −289 showed reduced responsiveness to sucrose alone. These data indicate that a sucrose response element may lie between positions −165 and −289.

Further Resolution of a MeJA-Responsive DNA Domain

Deletion of sequences between positions −787 and −520 rendered the *vspB* promoter noninducible by MeJA, suggesting that a MeJA-responsive domain was located in this region. A comparison of this DNA sequence to *chs*, *vspA*, and proteinase inhibitor II promoters (all of which are MeJA inducible) revealed a 50-bp domain common to these genes. This domain, shown in Figure 9, contains a G-box motif (CACGTG) and a C-rich sequence. The 50-bp domain (−585 to −535) was fused to the −88 or −44 CaMV 35S truncated promoter and used to transform tobacco. Transformants containing only the −88 CaMV 35S promoter were minimally responsive to sucrose or MeJA (Figure 8). In contrast, transformants with the 50-bp DNA domain from *vspB* fused to the −88 CaMV 35S truncated promoter were induced sevenfold when leaves were treated with MeJA or when leaves were wounded (data not shown). The 50-bp domain fused to a −44 CaMV 35S promoter did not respond to MeJA, indicating that sequences in the −88 CaMV 35S promoter, perhaps the as-1 domain (binds activating sequence factor-1 [ASF-1]), were required for responsiveness to MeJA.

DISCUSSION

vspB Promoter Activity in Tobacco and Soybean

Soybean plants grown 4 weeks on a light/dark regime show high expression of *vspB* in young apical leaves, intermediate expression in internodes, and lower expression in mature leaves (Mason and Mullet, 1990). *vsp* expression within leaves is highest in the paraveinal mesophyll cells and bundle sheath cells, lower in epidermal cells, and lowest in mesophyll cells (Franceschi and Giaquinta, 1983). The expression of *vspB-gus* fusions in transgenic tobacco was similar but not identical to *vspB* expression in soybean. In 4-week-old tobacco plants, little *vspB*-driven *gus* expression was observed in young leaves, except in trichomes and the midrib. It is not known at present if *vspB* is expressed in soybean trichomes, but it is interesting to note that JA has been detected in trichomes of sagebrush (Farmer and Ryan, 1990). Higher levels of GUS activity were found in tobacco stems compared with young or mature leaves. Lower expression of the *vsp* promoter in tobacco leaves may be due to the absence of paraveinal mesophyll cells in tobacco. On the other hand, both transgenic tobacco and soybean show relatively high expression of the *vspB* promoter in stem tissue and both are stimulated by sucrose, MeJA, and wounding (Mason and Mullet, 1990; Mason et al., 1992). Also, the time scale of half-maximal *vsp* mRNA accumulation in soybean treated with MeJA (~12 hr, see Mason et al., 1992) is very similar to the half-maximal accumulation of GUS activity (~14 hr, data not shown) in *vsp-gus* transgenic tobacco. Therefore, although direct comparison of the endogenous *vspB* promoter to the *vspB-gus* constructs in tobacco is not simple, it is clear that many of the essential promoter elements controlling *vspB* expression in soybean are contained within the −787 to +46 region upstream of the *vspB* open reading frame.

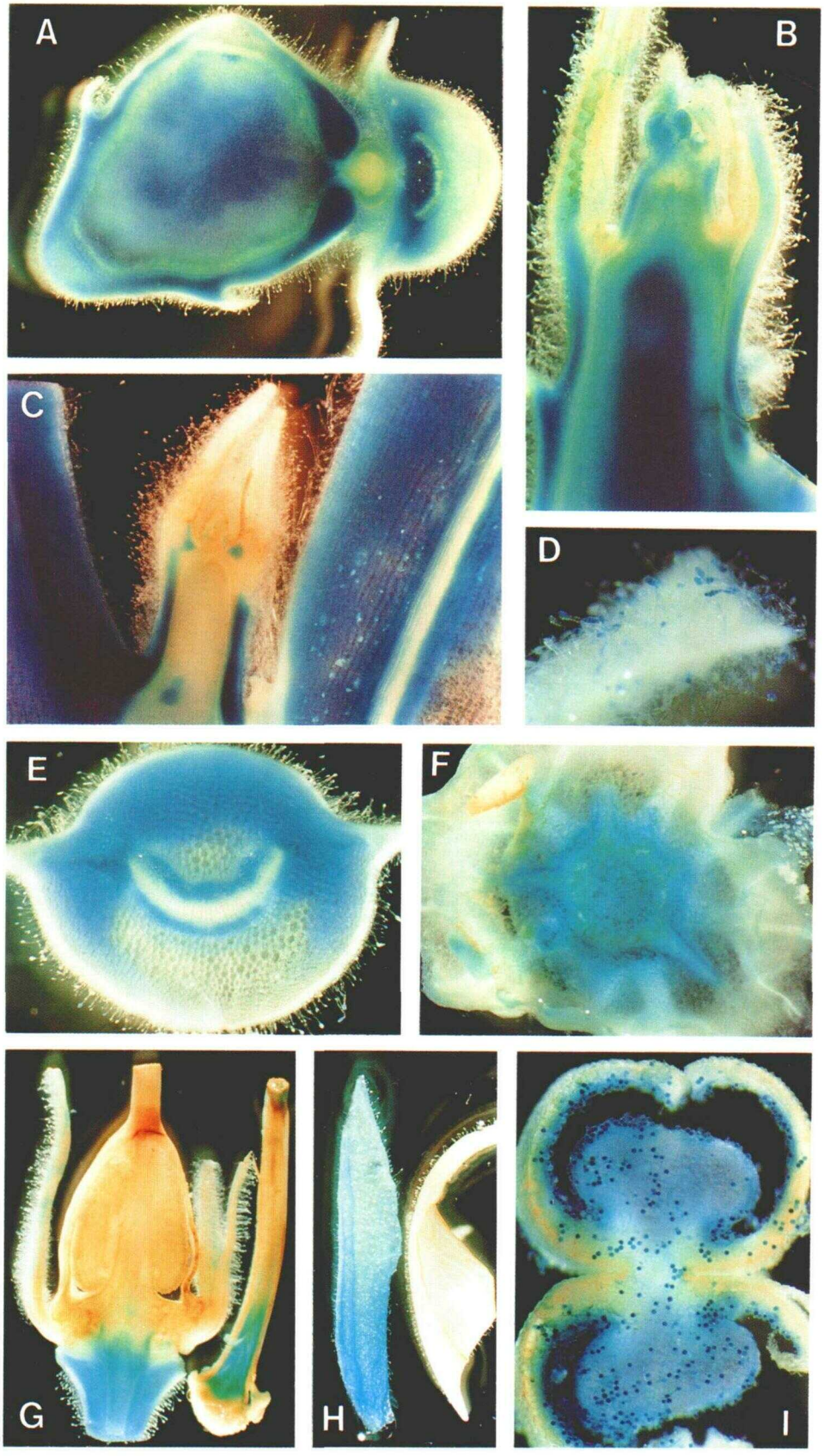

Figure 7. Histochemical Localization of GUS Activity in *vspB-gus* Transgenic Tobacco.

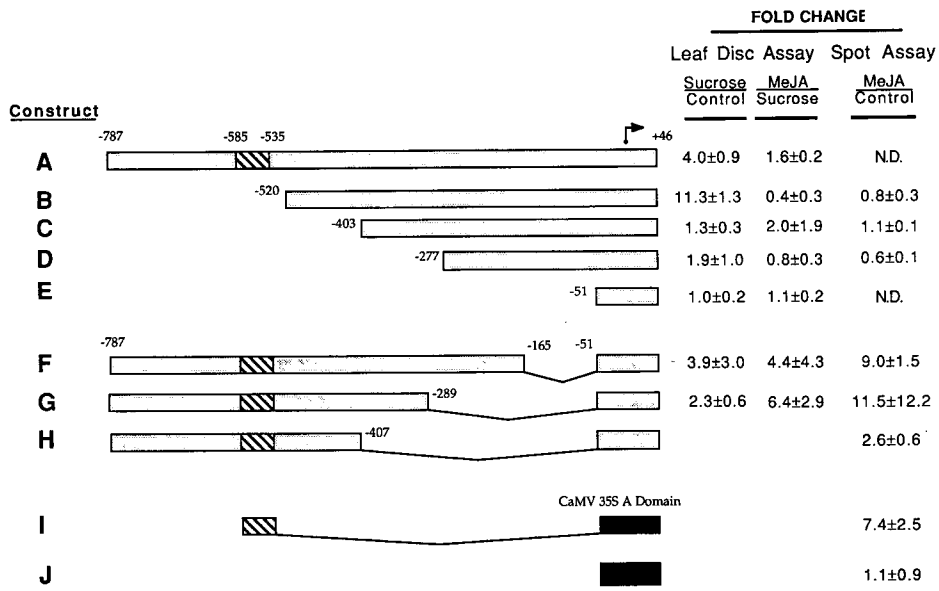

Figure 8. Deletions Alter the Response of *vspB-gus* Constructs to Sucrose and MeJA.

The 5′ end of the *vspB* promoter was deleted to the end points indicated, with a 3′ end at +46 (constructs A to E). The 3′ end of the *vspB* promoter was deleted to the end points indicated (constructs F to H) and fused to a *vspB* minimal promoter (−51 to +46). A 50-bp fragment from *vspB* (−585 to −535) was fused to a CaMV 35S minimal promoter (−88), which was fused to the 5′ nontranslated region of tobacco etch virus. These constructs were fused to the *gus* coding region and used to transform tobacco plants. Stippled boxes represent portions of the *vspB* promoter, and the hatched boxes represent the location of the MeJA-responsive domain in the promoter. Solid boxes represent the CaMV 35S (−88) minimal promoter. The bent arrow indicates the transcription start site. Two different assays were used to assess sucrose- and MeJA-induced *gus* expression. For the first assay (leaf disc assay), leaf pieces were cut from independent transformants growing in soil and treated, as given in Figure 5, with Murashige and Skoog (MS) salts (Control), MS salts plus 0.2 M sucrose (Sucrose), or MS salts plus 0.2 M sucrose plus 10 μM MeJA for 24 hr in the dark, then extracted and assayed for GUS activity. The second assay (spot assay) was performed, as described in Figure 1. Values are means ± SD of the ratios of the GUS activities of sucrose/control, MeJA/sucrose, and MeJA/control for two to six independent transformants of each construct. The mean basal activities expressed as picomoles per minute per milligram of protein for the different *vspB-gus* fusions are as follows: construct A, 80.0 ± 11.4; construct B, 11.2 ± 7.8; construct C, 24.7 ± 35.9; construct D, 6.6 ± 2.9; construct E, 3.2 ± 1.1; construct F, 19.1 ± 12.6; construct G, 8.9 ± 5.5; construct H, 22.3 ± 11.6; construct I, 33.4 ± 15.1; and construct J, 23.3 ± 17.1. Gene fusions in the figure are lettered arbitrarily; therefore, the construct labeled "A" is equivalent to the H construct in Figures 2 to 6. N.D., not determined.

Response to Sucrose

Several genes have been reported to be responsive to sucrose, including those that encode patatin (Wenzler et al., 1989; Jefferson et al., 1990), sporamin (Hattori et al., 1990), CHS (Tsukaya et al., 1991), and PI-II (Johnson and Ryan, 1990; Kim et al., 1991). In soybean cell culture, MeJA-induced *vspB* expression was stimulated by sucrose, glucose, or fructose with maximal stimulation occurring between 0.06 and 0.1 M (Mason et al., 1992). Similarly, treatment of soybean stems or leaves

Figure 7. (continued).

R_1 plants from transformant H2 were grown in soil and tissues were sampled by hand sectioning with a razor blade. The sections were stained for GUS activity as described by Jefferson et al. (1987), cleared with 70% ethanol, and photographed. Regions where GUS is localized appear blue.

(A) Transverse section through the stem (left) and axillary bud and subtending petiole (right).
(B) Longitudinal section through the stem and apical bud.
(C) Longitudinal section through the stem (left) and axillary bud and subtending petiole (right).
(D) Leaf piece showing staining of trichomes.
(E) Transverse section through a leaf petiole (adaxial side up).
(F) Transverse section through a flower pedicel.
(G) Longitudinal section through an ovule (left) and stigma/style (right).
(H) Flower sepal (left) and petal (right).
(I) Transverse section through an anther showing staining of pollen grains.

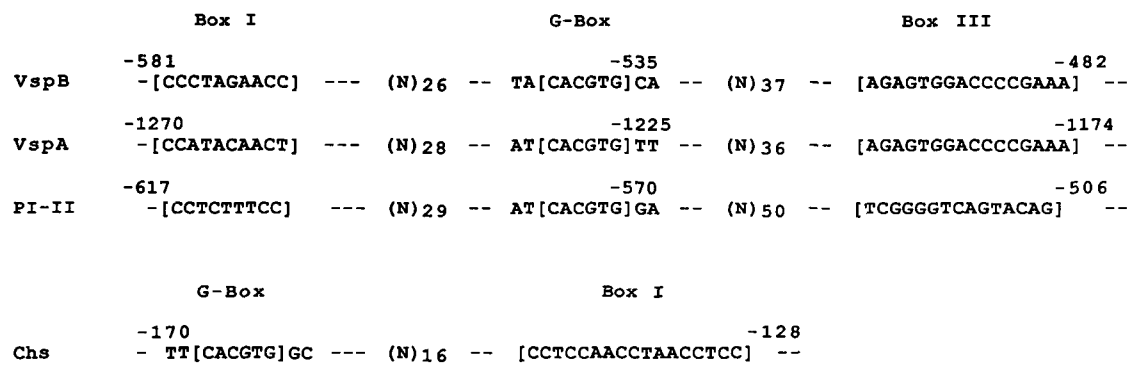

Figure 9. Comparison of Promoter Sequences from MeJA-Responsive Genes.

Alignment of conserved domains in 5′ promoters for vegetative storage protein genes (*vspA* and *vspB*), the potato proteinase inhibitor II (PI-II) gene, and the parsley chalcone synthase (*chs*) gene is shown. Numbering is from the transcription start site.

with sucrose increased *vsp* mRNA levels (Mason et al., 1992). Transgenic tobacco plants harboring the −1451 or −787 *vspB* promoter–*gus* construct showed increased expression when excised leaves were allowed to take up sucrose through their petioles over those in water alone (Figure 2). Excised leaf sections floated on Murashige and Skoog medium plus sucrose also showed elevated expression relative to water controls. Maximal stimulation by sucrose occurred at 0.2 M (Figure 4): a response similar to that observed with the patatin promoter (Wenzler et al., 1989) but higher than sucrose levels required to maximally stimulate the potato *pin2* promoter in tobacco (Johnson and Ryan, 1990) or *vsp* in soybean cell culture (Mason et al., 1992). The concentration of GUS activity in the phloem and stem nodes near the axillary buds is consistent with responsiveness of *vspB* to sucrose. The stem node is a site for temporary storage of carbon and nitrogen under some conditions. A similar localization of GUS activity in transgenic tobacco stems was seen with fusions of the sucrose-inducible spoA1 (sporamin) promoter and *gus* (Ohta et al., 1991).

Deletion of the 5′ end of the *vspB* promoter from positions −787 to −520 removed DNA sequences involved in response to MeJA but did not inhibit the response to sucrose. Further 5′ end deletion to position −403, however, significantly reduced the response to sucrose. Interestingly, constructs with 5′ deletions to −277 restored a small response to sucrose. Evidence for a role of DNA in the −165 to −289 region was also obtained with 3′ end deletions, because removal of these segments attenuated the sucrose response. These data suggest that multiple elements between positions −520 and −165 are responsible for modulation of *vspB* expression in response to sucrose. DNA regions in *chs* (Tsukaya et al., 1991), spoA1 (Hattori and Nakamura, 1988; Ohta et al., 1991), and patatin (Jefferson et al., 1990) have been implicated as *cis* elements that mediate responses to sucrose. Further analysis of the *vspB* promoter will be required to determine if similar sequence elements mediate *vspB* response to sucrose.

Identification of a MeJA-Responsive Domain

Transgenic tobacco plants harboring *vspB* promoter–*gus* constructs showed increased expression when plants were treated with MeJA (10^{-8} to 10^{-5} M). Induction was observed when MeJA was fed to excised leaf pieces or when applied to leaf surfaces. Deletion of 5′ end sequences from positions −787 to −520 relative to the transcription start site removed DNA that mediates MeJA-responsive expression. It should be noted, however, that the construct ending at position −520 was strongly activated by sucrose and that treatment with MeJA and sucrose together partially suppressed the induction. Furthermore, removal of sequences between −289 and −407 decreased the extent of stimulation of expression by MeJA. These results suggest that a part of the MeJA response complex is retained within the −520 construct and that the upstream elements between −520 and −787 interact with sequence elements/factors located between −289 and −407.

To further define sequences in the upstream region, a 50-bp DNA domain (−535 to −585) was fused to the −88 CaMV 35S promoter. The −88 CaMV 35S promoter contains a TATA element and an *as-1* element, which binds ASF-1 (Lam et al., 1989). Alone, the −88 CaMV 35S promoter is minimally responsive to MeJA; however, when combined with the 50-bp domain from *vspB*, the promoter was stimulated sevenfold by the addition of MeJA. The 50-bp MeJA-responsive domain contains a hexameric G-box motif (for review, see Williams et al., 1992) (CACGTG) located in the promoters of numerous genes and a C-rich sequence previously noted upstream of wound-inducible genes (phenylalanine ammonia-lyase [*pal*] and *chs*)

(Schulze-Lefert et al., 1989; Staiger et al., 1989). Because *vspA* and *vspB* are both responsive to JA/MeJA, we searched for a similar domain in *vspA*. As shown in Figure 9, *vspA* contains a similar sequence, although it is located further from the transcription initiation site compared to *vspB*. During the search, we noted a conserved sequence (box III, Figure 9) immediately downstream from the G-box in both *vspA* and *vspB*.

A G-box motif in the PI-II promoter has recently been found to be involved in MeJA-mediated responses (Kim et al., 1992). Similar to *vspA* and *vspB*, the G-box motif in PI-II is flanked by a C-rich domain (box I) and a box III–like sequence in reverse orientation. Furthermore, *chs*, which is responsive to MeJA (Creelman et al., 1992), elicitors (Ryder et al., 1984), sucrose (Tsukaya et al., 1991), and UV illumination (Ohl et al., 1989), also contains a G-box motif flanked by a C-rich sequence (box I, Figure 9) (Schulze-Lefert et al., 1989). The G-box and box I sequences are required, and together are sufficient to mediate UV induction of *chs* (Schulze-Lefert et al., 1989). Currently, it is not known if this same domain will mediate responses to JA/MeJA or if UV treatment increases JA/MeJA levels in plants. The G-box motif binds to a family of factors of the basic/leucine zipper (bZIP) class of proteins (Staiger et al., 1989; for review, see Williams et al., 1992). These *cis*-element/*trans*-factor complexes often interact with other DNA–protein complexes to modulate gene expression. It seems likely that the G-box/factor complex implicated in JA/MeJA responses acts in concert with other factors to mediate selective activation of *vspB* in response to elevated JA/MeJA levels. Further analysis of the *vspB* promoter and interacting *trans* factors will be required to clarify how JA/MeJA and carbon status interact to modulate *vsp* gene expression.

METHODS

Plant Culture Conditions

Soybean (*Glycine max* cv Williams 82) plants were grown in vermiculite in the dark at 28°C.

Genomic DNA Isolation and Cloning

Genomic DNA was isolated from 3-day-old dark-grown soybean seedlings as follows. Ten grams of tissue was ground to a powder in liquid N_2 and transferred to a 400-mL beaker containing 50 mL of buffer (0.1 M Tris-HCl, pH 8.0, 20 mM EDTA, 0.25 M NaCl, 0.3 mg mL^{-1} proteinase K) preheated to 55°C. RNase A and *n*-lauryl sarcosine were added to the slurry to final concentrations of 20 $\mu g/mL^{-1}$ and 1%, respectively, and the slurry was shaken at 65 rpm and incubated at 55°C for 2 hr. Then, SDS was added to the mixture to a final concentration of 1% and the mixture was shaken for 0.5 hr. The nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), then extracted with chloroform/isoamyl alcohol (24:1), and precipitated with 0.3 M NaOAc and 0.6 volume of 2-propanol. The nucleic acid was resuspended and the DNA was banded in CsCl. The DNA was partially digested with Sau3AI to yield an average molecular length of 15 kb, partially filled in with dGTP and dATP using the Klenow fragment of DNA polymerase I, and ligated with λGEM-11 XhoI half-site arms (Promega). The ligated products were packaged using Gigapack Plus (Stratagene) according to the manufacturer's directions. The primary genomic library was screened using *Escherichia coli* KW251 host cells and probed with a single-stranded RNA probe that was complimentary to cDNAs encoding VSPα (pKSH1) and VSPβ (pKSH3) (Mason et al., 1988).

DNA Sequencing and Transcript Mapping

A 1534-bp XbaI-PstI genomic fragment containing sequences upstream from the *vspB* coding region and part of the N-terminal coding region was subcloned into pBluescript KS(+) (Stratagene) to make pKS-vspBXP. The 5′ end of the XbaI-PstI fragment (−1451) was slightly shorter than that previously reported (−1455) (Rhee and Staswick, 1992b). Unidirectional deletions were obtained from the 5′ and 3′ ends of this fragment using exonuclease III and mung bean nuclease, as described by the manufacturer (Stratagene). Dideoxy sequencing of these deletions was performed using a Sequenase kit (U.S. Biochemicals). The 5′ end of the *vspB* mRNA was determined by primer extension and RNase mapping (Sambrook et al., 1989), and confirmed the end point observed by Rhee and Staswick (1992b).

Vector Construction and Plant Transformation

The *vspB* coding region sequences were deleted from pKS-vspBXP to a position 7 bp upstream of the initiation codon (+46) using exonuclease III and mung bean nuclease. The resulting fragment was ligated into XbaI/SmaI–digested pBI101 (Clonetech, Palo Alto, CA) upstream of the *gus* coding region to make pBI101-X. A 5′ deletion of 665 bp was obtained by digesting pBI101-X with HindIII and religating at low DNA concentration to make pBI101-H. A 576-bp fragment from the 3′ end of *vspB* (+1768 to +2344) was obtained by polymerase chain reaction (PCR) using the parent λ clone as a template. The primers incorporated a SacI site at the 5′ end and a EcoRI site at the 3′ end. The PCR product was digested and ligated into pBI101-X in place of the nopaline synthase (*nos*) terminator to yield pBI101-XV.

Deletions of the 5′ end of the *vspB* fragment in pBI101-X were obtained by exonuclease III/mung bean nuclease digestion at the XbaI site, with upstream sequences being protected by previous PstI digestion. Deletions of the 3′ end of the *vspB* fragment in pKS-vspBXP were obtained as above, with the fragments isolated after truncation at the HindIII site at position −787 and blunt ended at the 3′ end, and ligated to pBI101-H, which had been digested with BglII and mung bean nuclease followed by HindIII.

A 50-bp fragment from the *vspB* promoter (−585 to −535) was duplicated by annealing synthetic complimentary oligonucleotides and ligated upstream of cauliflower mosaic virus (CaMV) 35S minimal promoters (−88 or −44), which were fused to the 5′ nontranslated leader of tobacco etch virus (Carrington and Freed, 1990), which was fused to the *gus* coding region in pBI101. These plasmids, and pBI121 (Clonetech), containing the CaMV 35S promoter upstream of GUS were used to transform *Agrobacterium tumefaciens* LBA4404 by the direct method (An et al., 1988). Plasmid structure in *A. tumefaciens* was confirmed by restriction digestion of plasmid prepared from cultures by alkaline lysis. Tobacco (*Nicotiana tabacum* cv Samsun) was transformed

by the leaf disc method (Horsch et al., 1988). Kanamycin-resistant callus was generated on media containing 200 μg/mL kanamycin and 200 μg/mL cefotaxime, and shoots were rooted in media containing 100 μg/mL kanamycin. Rooted plantlets were transferred to soil and maintained in growth chambers on half-strength Hoagland's medium.

Plant Treatments, Determination of GUS Activity, and Histochemical Staining

Mature leaves were excised from tobacco plants, their petioles placed into different media, and incubated in the light for 24 hr. Tissue from the basal one-third of the lamina (excluding the midrib) was sampled for determination of GUS activity. Alternatively, mature leaves were excised, surface sterilized with 10% bleach/0.05% Tween-20 for 10 min, washed four times with distilled water, and cut into pieces ~1 cm^2, excluding lateral veins and midrib. The leaf pieces were floated for 24 hr on Murashige and Skoog medium (Sigma) plus B5 vitamins with or without additions, as noted in legends to Figures 3 to 5. The leaf pieces were blotted dry and frozen at −80°C for later determination of GUS activity. GUS activity was measured by fluorometric assay (Jefferson et al., 1987). Tissues were homogenized in GUS lysis buffer (Jefferson et al., 1987) and soluble material obtained by microcentrifuging at 12,000*g* for 5 min. Protein was measured by Coomassie G-250 dye-binding assay (Bradford, 1976). GUS activity is expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

Sections of tissues were obtained by hand with a razor blade and incubated in 50 mM NaP_i/1 mM X-gluc (Jefferson et al., 1987) at 37°C for 16 hr. The tissues were cleared with 70% ethanol.

ACKNOWLEDGMENTS

We thank Sharyll Pressley for her help in assembling this manuscript. We thank Jim Carrington for the tobacco etch virus DNA. This research was supported by the Texas Agricultural Experiment Station, the Texas Advanced Research Technology Program, and Grant No. 91-37304-6658 from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program.

Received November 10, 1992; accepted January 11, 1993.

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