

Expression of Two Soybean Vegetative Storage Protein Genes during Development and in Response to Water Deficit, Wounding, and Jasmonic Acid

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The expression of *vspA* and *vspB* genes encoding soybean vegetative storage proteins was studied during seedling development and in response to water deficit, tissue wounding, and jasmonic acid treatment. *vspA* and *vspB* encode VSP- α and VSP- β , 28-kilodalton and 31-kilodalton vacuole-localized polypeptides that are 80% homologous. *vspA* and *vspB* mRNAs could be distinguished on RNA blots using 3'-end probes. *vspA* mRNA was threefold to sevenfold more abundant than *vspB* mRNA in leaves, about equal expression was observed in stems, and *vspB* mRNA exceeded *vspA* in roots. Transcripts were not detected in dry seeds but appeared in intact or excised seedling axes between 12 hr and 24 hr after initiation of imbibition. Both transcripts were highly abundant in the meristematic region of seedling stems and in developing leaves but were rare in mature stems, leaves, and roots. In situ localization showed that *vsp* transcripts were found throughout the hypocotyl hook but were concentrated in cells associated with the epidermis and vascular bundles. Water deficit caused increased *vsp* mRNA levels in leaves and stems, which suggests that inhibition of growth necessitates temporary storage of amino acids. Wounding induced primarily *vspB* mRNA in etiolated seedlings, whereas both *vspA* and *vspB* mRNA levels increased in wounded leaves. Jasmonic acid and methyl jasmonate were potent inducers of *vsp* gene expression in cell cultures, developing axes, leaves, and roots. We hypothesize that jasmonic acid levels modulate *vsp* mRNA abundance in vivo.

INTRODUCTION

We recently described two related glycoproteins that are abundant in apical growing tissues of stems of dark-grown soybean seedlings and cDNAs encoding them (Mason et al., 1988). The proteins have an apparent molecular mass of 28 kD and 31 kD on SDS-PAGE, are 80% homologous in amino acid sequence, and differ in net charge. The proteins are most abundant in the stem hook and elongating regions of dark-grown seedlings, and during growth at low water potential, the abundance of the 28-kD protein in cell walls and cytoplasmic fractions increases (Bozarth et al., 1987; Mason et al., 1988).

We now know that these same glycoproteins were earlier identified in soybean leaves and were termed vegetative storage proteins (VSP) (Wittenbach, 1983). Recently cDNA clones that encode the leaf VSP were characterized. The deduced leaf VSP amino acid sequences are identical to those obtained from soybean stem cDNAs (Mason et al., 1988; Staswick, 1988, 1989b). Therefore, we propose the names *vspA* for the gene encoding the VSP- α polypeptide, which has been reported to migrate between 25 kD and 28 kD on SDS gels, and *vspB* for the

gene encoding the VSP- β polypeptide, which has been reported to migrate between 27 kD and 31 kD (Wittenbach, 1983; Crafts-Brandner and Egli, 1987; Mason et al., 1988; Staswick, 1988; Anderson et al., 1989). In this paper we will refer to the gene transcripts as *vspA* mRNA or *vspB* mRNA.

VSP accumulate in vacuoles of leaf paraveinal mesophyll cells and bundle sheath cells before flowering, decline during early pod-fill, and reaccumulate during late pod-fill (Franceschi et al., 1983; Wittenbach, 1983; Staswick, 1989a). Removal of pods from soybean plants or petiole girdling to block phloem transport causes accumulation of the VSP as well as several other proteins in leaves (Wittenbach, 1983; Staswick, 1989a). With continued pod removal the VSP can accumulate and represent up to 45% of total leaf soluble protein. Analysis of *vsp* mRNA levels shows that changes in VSP protein accumulation are paralleled by changes in total *vsp* mRNA abundance (Mason et al., 1988; Staswick, 1989a). Immunological studies show that the VSP are found in leaves, flowers, pods, cotyledons of germinating seedlings, and stems but are very rare in seeds and roots (Staswick, 1989c). In addition, VSP- α is found to be more abundant in leaves than VSP- β , whereas VSP- β levels exceed VSP- α levels in roots

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and nodules (Staswick, 1989c). The VSP are also found to form α_2 , $\alpha\beta$, and β_2 dimers (Spilatro and Anderson, 1989).

The plant growth regulator jasmonic acid (JA) has been reported to stimulate the accumulation of VSP- β and several other proteins in soybean cell cultures (Anderson, 1988; Anderson et al., 1989). JA induces both VSP- α and VSP- β in soybean leaves (Anderson et al., 1989), and JA-methyl ester (JA-Me) induces specific changes in poly(A)⁺ RNA in barley leaf (Mueller-Uri et al., 1988). The ability of JA to regulate gene expression may be of general significance because this plant growth regulator has been reported in a large number of plants (Meyer et al., 1984). The ability of JA to induce VSP accumulation in soybean cell cultures at very low concentrations (1 μ M to 10 μ M) distinguishes its modulation of gene expression from its ability (at concentrations >50 μ M) to induce leaf senescence (Ueda and Kato, 1981), potentiate leaf abscission (Curtis, 1984), or inhibit pollen germination or the growth of rice seedlings (Yamane et al., 1981). The biosynthetic origin of JA from linolenic acid and its structural similarity to mammalian eicosanoids (Vick and Zimmerman, 1984) are consistent with an important role for JA as a lipid-derived regulator of plant metabolism and gene expression.

In this paper, we examined the expression of *vspA* and *vspB* genes during plant development and in response to water deficit, tissue wounding, and JA using probes that hybridize specifically with either *vspA* or *vspB* transcripts. Based on this data we propose a hypothesis to explain the expression of the *vsp* genes in young growing tissues and the modulation of gene expression by wounding and water deficit.

RESULTS

Probe Specificity

To measure the relative abundance of *vspA* and *vspB*, it was necessary to make probes that were specific for each transcript. This was done by subcloning the 3'-terminal noncoding regions of the cDNAs in which the homology between the clones was only 50%. Antisense RNA probes made from these clones were used to test hybridization to sense RNA made from the full-length clones. Figure 1 shows that the *vspA* probe hybridized only with *vspA* transcripts and the *vspB* probe only with *vspB* transcripts under the conditions specified. For purposes of comparison, hybridization signals were quantitated either by scanning the blot with a Betascope 603 (Betagen Corp., Waltham, MA), or by elution of silver grains from the x-ray films (Suissa, 1983).

vsp mRNA Distribution in Dark-Grown Seedlings

We previously published data showing a high abundance of VSP and their mRNAs in the apical hook of dark-grown soybean seedlings and decreasing abundance in the elongating and mature stem regions (Mason et al., 1988). Using the specific probes tested in Figure 1, we examined the levels of *vspA* and *vspB* mRNAs during germination and early seedling development. Figure 2 shows that the *vsp* mRNAs were very rare in dry seed and in the seedling axis 12 hr after imbibition, but by 24 hr after imbibition, they became quite abundant in the stem hook. After 3 days of seedling growth in the dark, *vsp* mRNAs were most abundant in the cotyledons, plumule, and stem hook, less so in elongating and mature stem and mature root, and very rare in root tips. In stem tissues *vspB* mRNA was about twofold more abundant than the *vspA* mRNA, whereas the opposite was true in the plumule, which contains embryonic leaves.

Because *vsp* transcripts are rare in dry seeds, we wanted to determine the time course for induction of *vsp* gene expression in the seedling axis during imbibition. In addition, we wondered whether JA-Me would alter this time course because VSP induction by JA in cultured soybean cells has been reported (Anderson et al., 1989). Imbibition was complete by 4 hr after initiation when axes were excised from the cotyledons. Axis elongation was observed between 12 hr and 24 hr in both control axes and those treated with 10 μ M (\pm)JA-Me. Figure 3 shows that *vsp* mRNA began to accumulate substantially in excised axes between 14 hr and 16 hr after initiation of imbibition, and *vspB* transcripts were 15-fold more abundant than *vspA* transcripts at this time. The level of *vspB* mRNA increased 13-fold between 14 hr and 16 hr, whereas *vspA* transcripts increased ninefold. If the excised axes were cultured with 10 μ M (\pm)JA-Me, the level of *vspB* mRNA at 12 hr was 27-fold higher than in untreated axes. However, JA-Me had little effect on the accumulation of

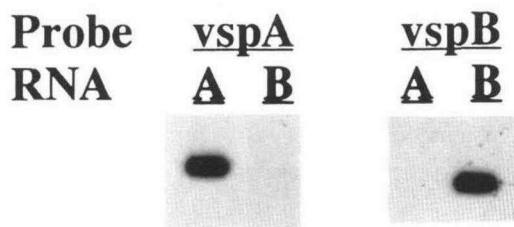


Figure 1. Specificity of Probes for *vspA* and *vspB* mRNAs.

Sense RNA encoding the VSP- α and VSP- β polypeptides were fractionated on formaldehyde-agarose gels, blotted to nylon membranes, and probed with antisense RNA complementary to the 3'-ends.

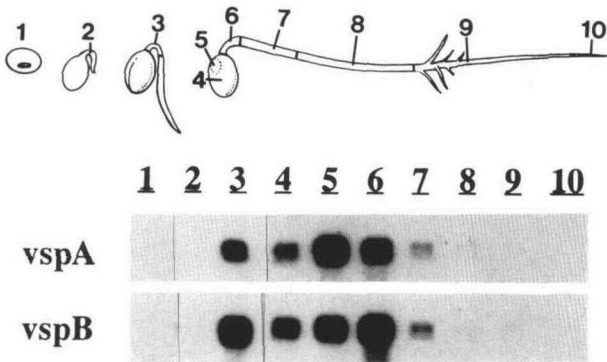


Figure 2. Distribution of *vspA* and *vspB* mRNAs in Dark-Grown Soybean Seedlings.

Probes specific for *vspA* and *vspB* mRNAs (Figure 1) were used to probe gel blots of total nucleic acid ($3.4 \mu\text{g}/\text{lane}$) from (1) dry seed, (2) 12-hr germinated seedling axis, (3) 24-hr germinated stem hook, and (4) cotyledon, (5) plumule, (6) stem hook, (7) stem-elongating region, (8) stem mature region, (9) root mature region, and (10) 1.5-cm root tip from 3-day-old dark-grown seedlings. Numbers above lanes correspond to the seedling stage or organ as numbered in the diagram.

vspA mRNA in excised axes during this time interval. Incubation of excised axes with $6 \mu\text{M}$ IAA had no effect on *vsp* mRNA levels, and treatment with $6 \mu\text{M}$ IAA + $0.5 \mu\text{M}$ kinetin inhibited *vsp* mRNA accumulation (H.S. Mason and J.E. Mullet, unpublished results).

When 2-day-old dark-grown seedlings are transplanted to vermiculite having low water potential (-0.3 MPa), stem growth decreases and the amount of VSP- α increases about twofold in stem hook and elongating regions (Bozarth et al., 1987; Mason et al., 1988). Figure 4 shows that *vspA* mRNA also increased about twofold in stem hook and elongating regions of stressed seedlings. *vspB* mRNA decreased in the hook and elongating regions in response to water deficit. The increase in *vspB* mRNA in the mature root during this treatment may be a response to transplant manipulation because this effect was not consistent. Wounding of dark-grown seedlings increased the abundance of *vspB* transcripts, especially in the mature stem and root tissues (Figure 4).

Because the ABA content of dark-grown soybean seedlings increases when plants are transplanted to -0.3 MPa vermiculite (Bensen et al., 1988), we wondered whether exogenous ABA applied to well-watered plants would induce *vspA* mRNA accumulation. When seedlings were transplanted to vermiculite containing $100 \mu\text{M}$ (\pm)ABA for 12 hr, the changes in *vsp* transcript levels were small and similar to the effects of low water potential (Figure 4). This treatment was found previously to increase internal ABA

levels above those found in the elongating stem region of seedlings exposed to -0.3 MPa vermiculite (Creelman et al., 1990). Thus, the small effects observed in ABA-treated plants may be due to the inhibition of growth rather than a direct modulation of gene expression. In contrast, when seedlings were transferred to vermiculite containing $5 \mu\text{M}$ (\pm)JA-Me, after 12 hr seedlings showed greatly increased levels of *vspB* mRNA, especially in mature root and stem tissues, whereas *vspA* mRNA levels increased less. At $20 \mu\text{M}$ (\pm)JA-Me, *vspA* and *vspB* levels increased further. Importantly, no inhibition of growth was observed with these JA-Me treatments, indicating that the effect of JA-Me may be more direct.

Illumination of dark-grown seedlings slows stem growth. RNA gel blot analysis in Figure 5 demonstrates that when 2-day-old dark-grown seedlings were transferred to an illuminated growth chamber, the abundance of *vsp* mRNA increased (measured here with cross-hybridizing, full-length cDNA probe) within 4 hr and was above the dark control after 8 hr.

Modulation of *vsp* mRNA Abundance in Light-Grown Plants

In well-watered, light-grown soybean plants, young growing leaves had the highest abundance of *vsp* mRNA and older mature leaves had lower levels, as shown in Figure 6. *vspA* mRNA was about threefold to sevenfold (depending on leaf position) more abundant than *vspB* mRNA in leaves. VSP- α is also more abundant than VSP- β in leaves (Staswick, 1989a). In stem internodes the pattern was somewhat different; whereas the youngest internode (6th) had the highest *vsp* mRNA levels, expression was lower in internode 4 than in older internodes 0 and 2 (Figure 6).

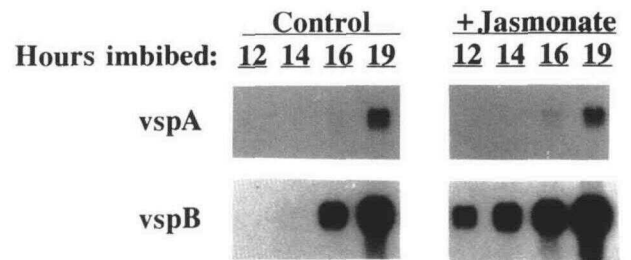


Figure 3. Induction of *vspA* and *vspB* mRNAs in Imbibing Seedling Axis.

Blots of total nucleic acid ($9 \mu\text{g}/\text{lane}$) were probed as in Figure 1. Soybean seeds were imbibed from time = 0 hr to 4 hr in water, then the seedling axes were excised from the cotyledons and incubation continued in the dark at 30°C in either 0.1 mM CaCl_2 or $10 \mu\text{M}$ (\pm)JA-Me/ 0.1 mM CaCl_2 . Times indicate hours after initiation (time = 0 hr) of imbibition.

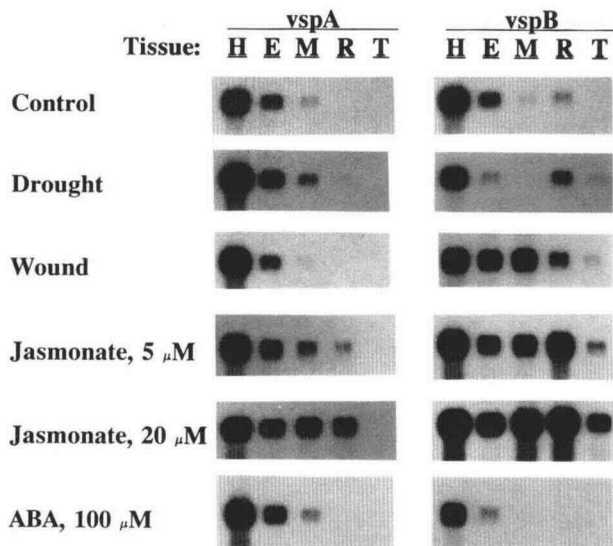


Figure 4. Modulation of *vspA* and *vspB* mRNAs in Dark-Grown Soybean Seedlings.

Total nucleic acid (3.4 $\mu\text{g}/\text{lane}$) from stem hook (H), elongating stem (E), mature stem (M), mature root (R), and 1.5-cm root tip (T) was probed as in Figure 1. Two-day-old dark-grown seedlings were transplanted to well-watered vermiculite (Control) or water-deficit vermiculite (Drought, water potential = -0.3 MPa) for 24 hr, cut into 2-mm to 3-mm pieces and incubated at 100% relative humidity for 12 hr (Wound) or transplanted to well-watered vermiculite containing 5 μM or 20 μM (\pm)JA-Me or 100 μM (\pm)ABA for 12 hr.

When water was withheld until the water potential of leaf 3 had decreased from -0.6 to -1.2 MPa, *vsp* mRNA levels were increased greatly in leaf 5 but only slightly in leaves 6 and 7, where expression was already quite high (Figure 6). In leaf 5 *vspA* and *vspB* mRNAs increased fivefold and tenfold, respectively, but *vspA* mRNA was still about twofold more abundant than *vspB*. Very little *vsp* mRNA induction was seen in mature leaves 1 and 3 during water deficit. In contrast, the mature stem internodes showed a much greater increase in *vsp* mRNAs in response to drought than did younger internodes. For example, both mRNAs increased about tenfold in internodes 0 and 2, whereas internode 6 showed only a twofold increase. Rewatering of droughted plants caused a nearly complete recovery of the water potential of leaf 3 after 1 day (-0.65 MPa). The recovery was also apparent in the *vsp* mRNA levels, which returned to lower abundance in both leaves and stem internodes (Figure 6). However, in leaf 5 *vspB* mRNA remained about sixfold higher than in the well-watered control, and in internodes 0 and 2 *vspA* mRNA was still elevated about fourfold over the well-watered control.

During studies on the induction of *vsp* gene expression in excised soybean leaves, we observed an apparent induction due to wounding. Because Staswick (1989a) reported that interruption of phloem transport by heat-killing of petiole phloem causes a great increase in *vsp* mRNAs, it is possible that the apparent effect of wounding could be due to inhibition of phloem transport. Thus, we devised an experiment whereby a single leaflet on an otherwise intact plant was wounded by cutting off 1 cm of the tip, a treatment which should not inhibit phloem transport from the wounded tissue still attached to the leaf base. This treatment caused a threefold to fourfold increase in both *vspA* and *vspB* transcripts in wounded tissue after 12 hr, but no change in *vsp* mRNAs was seen in more basal leaf tissue, as shown in Figure 7A. Excision of leaves and incubation in a dark humid box caused a slight increase in *vsp* transcript levels in all regions of the leaf blade, which is consistent with the finding that *vsp* mRNA levels increase in intact leaves during the dark period (Staswick, 1989a). Wounding of excised leaves again caused severalfold elevation of *vsp* mRNA abundance (Figure 7A). Induction of *vsp* mRNA by wounding of mature soybean leaves by crushing the distal third of a leaflet is shown by Staswick (1990).

Spraying (\pm)JA or (\pm)JA-Me at 30 μM on intact soybean leaves induced a threefold elevation of *vspA* mRNA in mature leaves within 12 hr after spraying (Figure 7B). Because penetration of leaf epidermis may be slow, we also excised mature leaves and allowed uptake of 10 μM (\pm)JA-Me through the cut ends for 12 hr. This treatment caused a similar increase in levels of *vsp* mRNAs as did spraying, again with *vspA* mRNA the predominant species

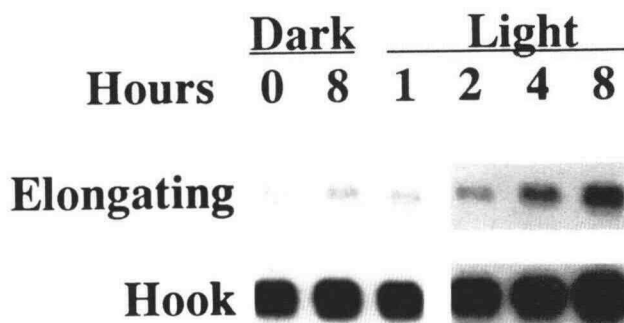


Figure 5. Effect of Light on *vsp* mRNA in Dark-Grown Soybean Stem.

Total nucleic acid (5.4 $\mu\text{g}/\text{lane}$) was probed with antisense RNA made from full-length *vspA* cDNA, which also hybridizes with *vspB* mRNA. Two-day-old dark-grown soybean seedlings were either maintained in the dark for 8 hr or transferred to growth chamber light for 8 hr. At the hours indicated, stem hooks and elongating regions were harvested.

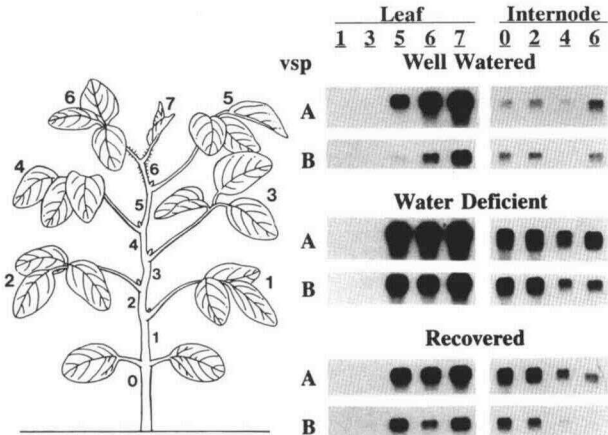


Figure 6. Distribution of and Modulation by Water Deficit of *vspA* and *vspB* mRNAs in Light-Grown Soybean.

Total nucleic acid from leaves and internodes ($4.1 \mu\text{g}/\text{lane}$) was probed as in Figure 1. Soybean plants were grown in controlled environment growth chambers until the seventh trifoliolate leaf was about 1 cm long, then water was withheld for 5 days and water-deficient plants were harvested. Recovered plants were watered at this time and harvested after 1 day. Well-watered plants were harvested at the same developmental stage as water-deficient plants. Numbers above lanes correspond to the leaf or internode number as shown on the diagram at left.

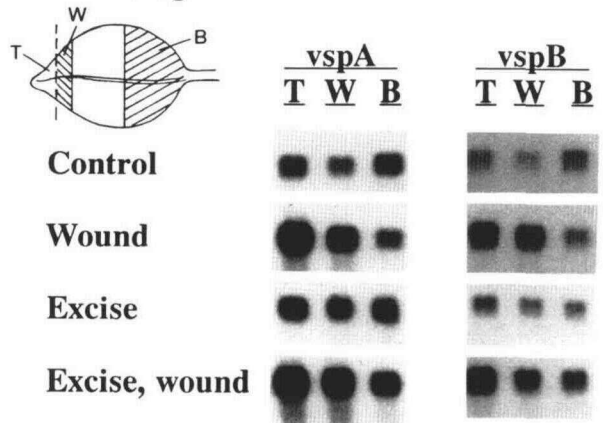
(Figure 7B). In contrast, treatment of photomixotrophically cultured soybean cells with $10 \mu\text{M}$ (\pm)JA-Me (or JA, data not shown) induced only *vspB* mRNA (Figure 8), which is consistent with the finding that only VSP- β is induced by JA in this system (Anderson et al., 1989). A 1.5-fold induction was apparent within 1 hr of initiation of treatment, and *vspB* mRNA accumulated progressively over a 24-hr period to a level that was tenfold greater than in untreated cultures. JA-Me was a better inducer of *vsp* mRNA than JA by about twofold (H.S. Mason and J.E. Mullet, unpublished results). This may be because of poor penetration of the acid, which would be about 50% ionized at the pH of the culture medium. In contrast, addition of Asn or Glu at 10 mM to soybean cell cultures had no effect on *vsp* mRNA levels (H.S. Mason and J.E. Mullet, unpublished results).

Histological Localization of *vsp* mRNAs

The VSP are localized in vacuoles of the paraveinal mesophyll and associated bundle sheath cells in soybean leaves (Franceschi et al., 1983). It was of interest to determine the histological localization of *vsp* transcripts in stems of dark-grown seedlings because stem anatomy is different from that of leaf. Localization of *vsp* mRNA by in

situ hybridization in stem sections is shown in Figure 9. By using ^{35}S -labeled, antisense RNA complementary to *vspA* mRNA, we probed tissue sections of 6-hr imbibed seedling axis, 26-hr imbibed stem hook, and stems of 3-day-old dark-grown seedlings. This probe hybridizes with *vspB* mRNA as well as *vspA* mRNA. The results indicated that the mRNA was absent or extremely low in the 6-hr axis but was discernible in the periphery of the 26-hr stem

A. Wounding



B. Jasmonate Stimulation

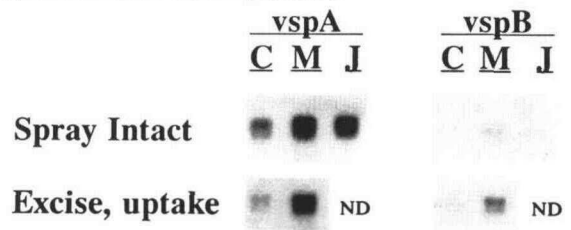


Figure 7. Modulation of *vspA* and *vspB* mRNAs in Soybean Leaves by Wounding and Jasmonate Treatments.

Total nucleic acid ($4.1 \mu\text{g}/\text{lane}$) was probed as in Figure 1. Soybean plants were grown as in Figure 6 and treated as follows:

(A) The third trifoliolate leaf was either left on the plant (Control, Wound) or excised at the petiole and stored in a dark humid box (Excise; Excise, wound). Wounding was accomplished by cutting off the terminal 1 cm of the middle leaflet with a razor blade. After 12 hr, the leaflet was dissected into the terminal 1 cm (T), the next 0.5 cm adjacent to the wound (W), and the basal 3 cm (B).

(B) Individual plants were sprayed with 0.05% Tween 20 (C), $30 \mu\text{M}$ (\pm)JA-Me/0.05% Tween 20 (M), or $30 \mu\text{M}$ (\pm)JA/0.05% Tween 20 (J) at the end of the photoperiod. After 12 hr in the dark, the third trifoliolate leaf was harvested. Alternatively, the third trifoliolate was excised under water from a single plant, and individual leaflets were incubated in the dark with their cut ends in water (C) or $10 \mu\text{M}$ (\pm)JA-Me (M). ND, uptake of JA was not done.

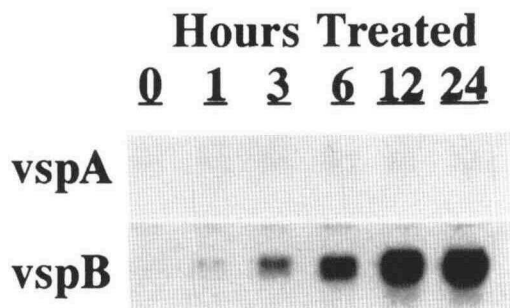


Figure 8. Induction of *vspB* mRNA by JA-Me in Soybean Suspension Cultured Cells.

Total nucleic acid (5.4 $\mu\text{g}/\text{lane}$) was probed as in Figure 1. On the fifth day after passage, a culture was made to 10 μM (\pm)JA-Me. Aliquots of the culture were harvested at the times (hours) indicated after stimulation with JA-Me.

hook. In the 3-day-old sample, heavy staining was apparent in the epidermis and adjacent cell layers and also in tissue associated with the vascular cylinder (Figures 9C, 9D, and 9E).

DISCUSSION

The soybean VSP were so named because of their localization in vacuoles of paraveinal mesophyll cells and accumulation in leaves in response to depodding (Franceschi et al., 1983; Wittenbach, 1983). More recently, these same proteins were found to accumulate in soybean hypocotyl hooks, young leaves, internodes, flowers, and seedpods (Mason et al., 1988; Staswick, 1989c). Other functions for the VSP have not been excluded and should be anticipated. Vegetative storage proteins in other plants have enzymatic activity or carbohydrate binding functions. For example, patatin, a vegetative storage protein in potato, is a lipid acyl hydrolase (Andrews et al., 1988), and bark and leaf lectins of *Sophora japonica*, a leguminous tree, are found in protein storage vacuoles (Herman et al., 1988). However, unlike soybean VSP, patatin is normally highly expressed in tubers but not in leaves. In this regard, the soybean VSP are more similar to a pea lectin-like protein that accumulates primarily in shoot apices (Dobres and Thompson, 1989). Although no hemagglutinating activity was detected in VSP preparations (Spilatro and Anderson, 1989), a lectin-like activity cannot be ruled out. If the VSP serve solely a storage function, it is not clear why the ratio of *vspA* and *vspB* expression varies in different tissues. For example, *vspA* mRNA is threefold to sevenfold more abundant than *vspB* in leaves, about equal mRNA levels are found in internodes of light-grown plants, and *vspB* mRNA levels exceed *vspA* mRNA levels in roots. In gen-

eral, VSP- α and VSP- β levels parallel *vspA* and *vspB* mRNA abundance (Staswick 1989a; this paper).

The two *vsp* genes also showed differential responses within the same tissue. During seed germination *vspB* mRNA began to accumulate 2 hr earlier than *vspA* mRNA and at least 4 hr earlier with jasmonate stimulation. Dark-grown seedlings that were exposed to mild drought by transfer to -0.3 MPa vermiculite for 1 day showed a doubling of *vspA* mRNA in stem hooks and elongating regions, whereas *vspB* mRNA decreased slightly. In root tips of dark-grown seedlings, *vspB*, but not *vspA*, mRNA was induced by wounding and jasmonate treatments. When light-grown plants were moderately stressed, levels of both *vsp* mRNAs increased over well-watered controls, but in this case *vspB* mRNA underwent a greater increase. Finally, a portion of the VSP- α protein co-purified with cell wall fractions, whereas most of the VSP- α and VSP- β protein is soluble (Mason et al., 1988). The significance of the cell wall-localized VSP is not understood.

Gene Expression, Development, and Jasmonic Acid

Expression of the *vsp* genes is complex and varies with respect to organ and cell type, developmental stage, and in response to wounding and drought. In nonstressed plants *vsp* mRNA levels (and protein levels) are highest in young developing organs of the shoot (stem, leaves, flowers, pods) and lower in differentiated nongrowing shoot tissues and roots. For example, *vsp* mRNA levels are highest in the hypocotyl hook, a meristematic region, lower in the stem zone of cell elongation, and very low in nongrowing cells of the lower stem (Mason et al., 1988). Likewise, immature leaves such as the plumule of the germinating seed or apical leaves of older plants have high *vsp* mRNA levels, whereas lower levels are found in mature leaves. Expression of the *vsp* genes is not restricted to the vegetative phase because VSP are present in flowers and seedpods (Staswick, 1989c).

The accumulation of VSP during the early phase of leaf, stem, or reproductive structure formation and their disappearance in differentiated tissues distinguishes these proteins from seed storage proteins and ribulose-1,5-bisphosphate carboxylase/oxygenase, which accumulate during seed and leaf maturation, respectively, as VSP levels decline. This observation suggests that the VSP accumulate at an early phase of cell development and serve as a source of amino acids for proteins that accumulate during cell maturation. Because activation of VSP synthesis creates a sink for amino acids in developing cells, it seems probable that induction of VSP synthesis is paralleled by increased uptake of amino acids such as Asn and Gln from the phloem. Furthermore, enzymes required to assimilate and convert transported amino acids (or amino acid precursors) into amino acid pools for protein synthesis are likely to be activated. Elevated glutamine synthetase levels

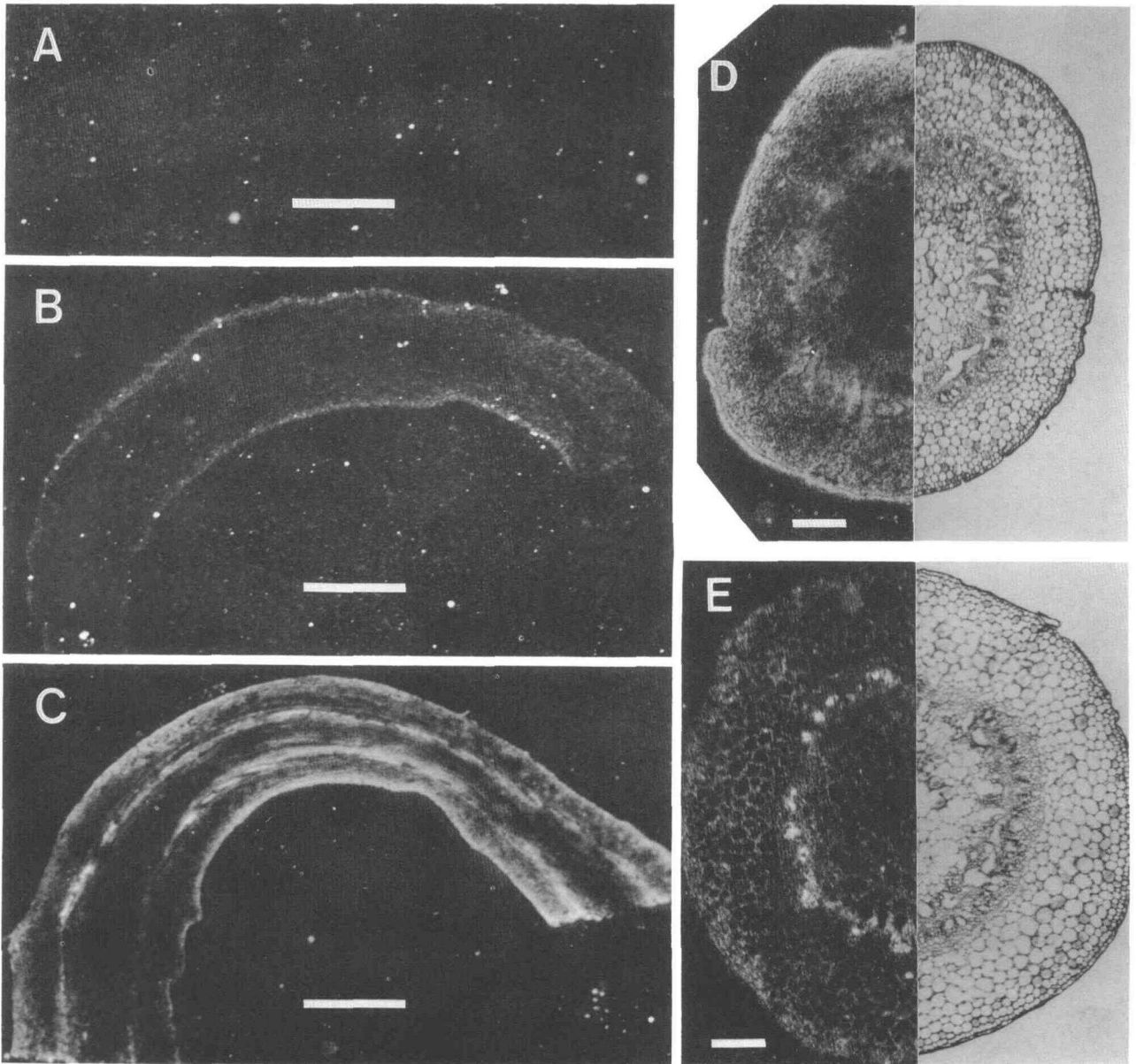


Figure 9. In Situ Localization of *vsp* mRNA in Soybean Seedling Stem.

Frozen sections of dark-grown soybean stems were probed with antisense RNA made using a full-length cDNA template encoding VSP- α .

(A) Longitudinal section of 6-hr imbibed seedling axis.

(B) Longitudinal section of stem hook from a seed imbibed for 26 hr.

(C) Longitudinal section of 3-day-old stem hook and part of elongating region.

(D) Transverse section of 3-day-old stem hook.

(E) Transverse section of 3-day-old stem elongating region.

In (A), (B), and (C), bar represents 1 mm. In (D) and (E), bar represents 0.2 mm, and right-side halves are different sections stained with toluidine blue.

in developing pea leaves is consistent with this idea (Edwards and Coruzzi, 1989).

The nature of the signal that induces VSP synthesis in developing soybean stems, leaves, and cell cultures was investigated in this paper and earlier by Anderson et al. (1989). We were able to modulate *vsp* mRNA levels in seedlings or cell cultures to only a small extent by altering ABA, IAA, kinetin, or amino acid availability (Asn or Gln). In contrast, JA and JA-Me were effective inducers of *vsp* mRNA in cell culture (*vspB* only), roots, stems, and leaves, which is consistent with VSP levels seen by Anderson et al. (1989). JA-Me also induced earlier accumulation of *vspB* mRNA in developing axes than observed *in vivo*. This could indicate that low expression of *vsp* genes in axes before 16 hr post-imbibition is due in part to low levels of JA. JA is present in soybeans (Meyer et al., 1984), but we lack important information concerning the relationship between JA levels and *vsp* gene expression. However, data from other plants are consistent with its proposed role as a modulator of *vsp* gene expression in soybean. For example, JA levels are high in immature leaves and stems and low in differentiated tissues (Knofel et al., 1984). Furthermore, in maize and sunflower, lipoxygenase and hydroperoxide dehydrase (enzymes involved in JA synthesis) are low in seeds and increase during early seedling growth, paralleled by increased levels of 12-oxo-phytyldienoic acid, a precursor of JA (Vick and Zimmerman, 1982).

We investigated the origin of the signal that induces *vsp* mRNA accumulation in hypocotyl hooks as well as the specific cells that accumulate *vsp* mRNA. Initially, we thought that the *vsp* inducer might arise from the cotyledons and be transported along with amino acids into the hypocotyl hook. However, when axes were separated from cotyledons at 4 hr post-imbibition, *vsp* mRNA appeared 12 hr later, similar to intact controls. These data suggest that *vsp* genes are induced by signals produced within the axis as a developmentally programmed event but do not rule out the cotyledons as a supplemental source of inducer. An alternate possibility is that excision of the cotyledons (wounding) induced *vsp* mRNA accumulation, but the similarity in timing of the induction with that seen in intact controls suggests otherwise. It appears that some competence for *vsp* gene expression is acquired by cells during post-germination development, e.g., a certain stage of cell development or the formation of specific cells. Because JA and JA-Me are potent inducers of *vsp* gene expression, this cellular competence may reflect a need to activate JA synthesis or accumulation, to alter the cells' sensitivity to JA, or to provide some other cell-specific factor. Cell-specific expression of *vsp* genes supports this view. *In situ* hybridization in hypocotyl sections shows *vsp* mRNA localization in epidermal cells, in cells near vascular bundles, and at lower levels in cortical cells. In leaves VSP proteins are found primarily in the paraveinal mesophyll, bundle sheath, and epidermal cells (Franceschi et al., 1983;

Staswick, 1990). In developing leaves the paraveinal mesophyll is the first tissue to differentiate (Franceschi and Giaquinta, 1983), which is consistent with its role in storage of reserve materials in immature leaves.

***vsp* mRNA Induction by Wounding**

We found that wounding increases *vsp* mRNA levels in leaves and dark-grown seedlings. Dissection of seedlings resulted in increased *vspB* mRNA accumulation primarily in nongrowing regions of stems and roots, but *vspA* mRNA levels were not altered significantly. Similar changes in *vsp* mRNA levels could be elicited by JA-Me. Furthermore, excision of a single 1-cm leaflet tip, which would have minimal effect on sink size and should not disrupt the movement of materials through the petiole, induced both *vspA* and *vspB* mRNA accumulation in tissue adjacent to the wound site but not in tissue distal to the wound site. This suggests that JA levels increase in or near the wounded cells, resulting in *vsp* mRNA accumulation. The first step in JA synthesis, lipoxygenase-catalyzed oxidation of linolenic acid, could be stimulated in wounded tissue. The rapid increase of 12-oxo-phytyldienoic acid (a precursor of JA) in wounded corn tissue is consistent with this possibility (Vick and Zimmerman, 1982). Other wound-induced signal molecules, such as pectic cell wall fragments (Bishop et al., 1984), cannot be ruled out.

Modulation of *vsp* Expression by Water Deficit

Transfer of 3-day-old dark-grown soybean seedlings to -0.3 MPa vermiculite resulted in inhibition of shoot growth and a twofold increase in VSP- α and *vspA* mRNA level. When older light-grown soybean plants were exposed to water deficit, stem and leaf growth were inhibited and *vspA* and *vspB* mRNA levels increased in several plant parts. Little increase was observed in older leaves, which showed very low *vsp* mRNA levels in control plants or in the youngest leaves where expression was already very high before water deficit. In contrast, large increases in *vsp* mRNA levels occurred in internodes and leaves that were partially expanded. When plants were rewatered, *vsp* mRNA levels adjusted toward prestress levels. One explanation for these observations can be derived from the proposed role of VSP as temporary storage proteins. During rapid vegetative growth, amino acids and other materials move from mature leaves and roots to the growing apex. Water deficit inhibits growth and, thus, utilization of these compounds, whereas it stimulates expression of the *vsp* genes and, presumably, the accumulation of VSP. Once water deficit is removed, amino acids in the VSP can be remobilized and used for leaf and stem growth. The same rationale can explain the increase in *vsp* mRNAs

during inhibition of hypocotyl elongation by light and ABA treatments.

This proposal is consistent with observed increases in VSP when flowers or pods are removed during the reproductive phase (Wittenbach, 1983; Staswick, 1989a). During reproductive development, leaf proteins are broken down and amino acids transported to the developing reproductive structures (Wittenbach, 1982). Thus, although removal of sinks results in accumulation of VSP in fully expanded leaves, we saw no accumulation of *vsp* mRNAs in fully expanded leaves with water deficit during the vegetative phase. It should be noted that reproductive structure development is very sensitive to water deficit, and we would expect water deficit during the reproductive phase to mimic the effect of flower/pod removal on VSP accumulation.

Conclusion

The soybean VSP serve a key role during the mobilization of amino acids from germinating seeds to developing stems and leaves and from mature leaves and stems to developing reproductive structures. The concentration of VSP and *vsp* mRNA appears to be modulated by the relative activities of source and sink tissues and therefore can be perturbed by water deficit. Results to date indicate that JA plays a central role in *vsp* gene expression. Further experiments will be required to confirm the role of JA in vivo, to elucidate its mode of action, and to understand how JA levels are regulated.

METHODS

Plant Material, Reagents

Soybean (*Glycine max* Merr cv Williams 82) seedlings were grown in the dark and dissected as described (Mason et al., 1988). Light-grown plants were grown in a growth chamber with 14-hr days (light intensity = $350 \mu\text{Em}^{-2} \text{sec}^{-1}$, day relative humidity = 50%, day temperature = 30°C, night temperature = 20°C) using Metro-mix 352 potting soil in 8-inch pots. Plants were watered as needed with half-strength Hoagland's medium and were droughted by withholding water. Plants were dissected as indicated in the figure legends. Suspension cultures of soybean were graciously supplied by Suzanne Rogers, Department of Horticultural Sciences, Texas A&M University, and grown photomixotrophically on PRB medium (Horn et al., 1983) containing 29 mM sucrose. Culture conditions were 23°C, ambient air, cool-white fluorescent illumination (fluence rate = $200 \mu\text{Em}^{-2} \text{sec}^{-1}$), and subculture on a 14-day cycle. Chemicals were obtained from Sigma and enzymes from Bethesda Research Laboratories, unless otherwise noted. (\pm)JA-Me was obtained from Bedoukian Research (Danbury, CT). (\pm)JA was prepared from (\pm)JA-Me by alkaline hydrolysis according to Anderson (1985).

Water Potential Measurement

Measurement of leaf and soil water potentials was performed by the isopiestic method as described by Boyer and Knippling (1965).

Probes

Subclones of pKSH2 and pKSH3, encoding VSP- α and VSP- β , respectively (Mason et al., 1988), and containing the 3'-terminal HindIII-EcoRI fragments were obtained in pBluescript (Stratagene). These fragments contain the 3'-terminal 29 nucleotides of coding and the 3'-untranslated regions of the full-length cDNAs, and are about 50% homologous over a length of 250 bp. Antisense RNA probes were made from these subclones using T3 RNA polymerase (Stratagene) according to supplier's protocols.

RNA Isolation and Hybridization

Total nucleic acid was isolated as described (Mason et al., 1988), except that only one phenol extraction was done. Total nucleic acid was quantitated by absorbance at 260 nm, denatured with formaldehyde, and fractionated in 1.2% agarose/4-morpholinepropanesulfonic acid/formaldehyde gels. In early experiments the uniformity of loading was assayed by ethidium bromide staining of gels, which showed equivalent amounts of RNA in each lane. The RNA was blotted to GeneScreen (Du Pont) nylon membranes in 25 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.5 and fixed by baking at 80°C for 2 hr. The blots were prehybridized as described (Mason et al., 1988), except that the buffer contained 50% formamide and $4 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}$, 0.015 M sodium citrate) and the temperature was 65°C. Hybridization was carried out in the same buffer containing $5 \times 10^5 \text{ cpm/mL}$ ^{32}P -labeled antisense RNA probes at 65°C for 12 hr to 16 hr. Blots were washed twice for 15 min in $2 \times \text{SSC}$, 0.5% SDS at room temperature and twice for 15 min in $0.1 \times \text{SSC}$, 0.5% SDS at 65°C, air dried, and exposed to Kodak X-AR film with an intensifier screen at -80°C. Signals were quantitated either by scanning blots with a Betascope 603 Blot Analyzer (Betagen Corp.), or by elution of silver grains from the x-ray films in 1 M NaOH (Suissa, 1983).

In Situ Localization

Localization of RNA and protein were performed on frozen sections of soybean stem. Cryostat sections 18 μm thick were picked up on poly-L-lysine-coated slides and dried at 45°C for 20 min. Sections were fixed in 4% paraformaldehyde, 50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.8, for 20 min at 25°C, dehydrated in 30%, 70%, 95%, and 100% ethanol, air dried, and stored desiccated until further use. RNA localization was performed by hybridization of sections with ^{35}S -labeled antisense RNA complementary to *vspA* mRNA, which had been hydrolyzed to an average length of 250 nucleotides. The prehybridization treatments were as described (Meyerowitz, 1987), and hybridization was carried out for 12 hr at 49°C in 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM Na-phosphate, pH 6.5, 5 mM EDTA, 10 mM DTT, 10%

(w/v) PEG-8000, 0.5 mg/mL yeast tRNA, and 0.02% each of BSA, Ficoll, and PVP, containing 1.5×10^5 cpm/ μ L of probe. Sections were washed 7 hr at 49°C in the same buffer without probe, equilibrated with RNase buffer (10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA), and digested with 20 μ g/mL RNase A 30 min at 37°C, washed with three changes of RNase buffer for 20 min each at 37°C, washed 12 hr at 49°C in hybridization buffer without probe, dehydrated through the ethanol series, and air dried. Slides were coated with NTB-2 emulsion (Kodak) diluted 1:1 with water, air dried, and exposed desiccated at 4°C for 3 days. Slides were developed in Kodak D-19 developer 5 min at 15°C, fixed 5 min, washed in water, stained 5 min in 0.05% toluidine blue, dehydrated in ethanol, cleared with xylene, and mounted in Permount.

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