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 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.
and nodules (Staswick, 1989c). The VSP are also found to form \( \alpha_2, \alpha_3, \) and \( \beta_2 \) dimers (Spilatro and Anderson, 1989). The VSP are also found to form \( \alpha_2, \alpha_3, \) and \( \beta_2 \) dimers (Spilatro and Anderson, 1989).

The plant growth regulator jasmonic acid (JA) has been reported to stimulate the accumulation of VSP-\( \beta \) and several other proteins in soybean cell cultures (Anderson, 1988; Anderson et al., 1989). JA induces both VSP-\( \alpha \) and VSP-\( \beta \) 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 \( \mu \)M to 10 \( \mu \)M) distinguishes its modulation of gene expression from its ability (at concentrations >50 \( \mu \)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 \( \mu \)M (±)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 \( \mu \)M (±)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.](image)

Sense RNA encoding the VSP-\( \alpha \) and VSP-\( \beta \) 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 µg/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 µM IAA had no effect on vsp mRNA levels, and treatment with 6 µM IAA + 0.5 µM 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 µM (±)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 µM (±)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 µM (±)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 µg/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 CaCl₂ or 10 µM (±)JA-Me/0.1 mM CaCl₂. Times indicate hours after initiation (time = 0 hr) of imbibition.
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 mRNA levels 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 (±)JA or (±)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 (±)JA-Me through the cut ends for 12 hr. This treatment caused a similar increase in levels of vsp mRNA as did spraying, again with vspA mRNA the predominant species.

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.

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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 μg/lane) was probed as in Figure 1. Soybean plants were grown in controlled environment growth chambers until the seventh trifoliate 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.

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 35S-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 μg/lane) was probed as in Figure 1. Soybean plants were grown as in Figure 6 and treated as follows:

(A) The third trifoliate 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 μM (±)JA-Me/0.05% Tween 20 (M), or 30 μM (±)JA/0.05% Tween 20 (J) at the end of the photoperiod. After 12 hr in the dark, the third trifoliate leaf was harvested. Alternatively, the third trifoliate 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 μM (±)JA-Me (M). ND, uptake of JA was not done.
mRNA levels exceed abundant than vspB in leaves, about equal mRNA levels and expression varies in different tissues. 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 Gin 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

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**DISCUSSION**

The soybean VSP were so named because of their localization in vacuoles of paraveinal mesophyll cells and accumulation in leaves in response to dehulling (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 general, VSP-α and VSP-β levels parallel vspA and vspB mRNA abundance (Staswick 1989a; this paper).
Vegetative Storage Protein Gene Expression

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 Gin). 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 (Knofl et al., 1984). Furthermore, in maize and sunflower, lipoygenase and hydroperoxide dehydrase (enzymes involved in JA synthesis) are low in seeds and increase during early seedling growth, paralleled by increased levels of 12-oxy-phytodienoic 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, lipoygenase-catalyzed oxidation of linolenic acid, could be stimulated in wounded tissue. The rapid increase of 12-oxy-phytodienoic 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 rewated, 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 µE m⁻² 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 µE m⁻² sec⁻¹), and subculture on a 14-day cycle. Chemicals were obtained from Sigma and enzymes from Bethesda Research Laboratories, unless otherwise noted. (+)JA-Me was obtained from Bedoukian Research (Danbury, CT). (+)JA was prepared from (+)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 formamide, and fractionated in 1.2% agarose/4-morpholinepropanesulfonic acid/formamide 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 NaH₂PO₄/Na₂HPO₄, 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 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate) and the temperature was 65°C. Hybridization was carried out in the same buffer containing 5 x 10⁶ cpm/mL 32P-labeled antisense RNA probes at 65°C for 12 hr to 16 hr. Blots were washed twice for 15 min in 2 x SSC, 0.5% SDS at room temperature and twice for 15 min in 0.1 x 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 NaH₂PO₄/Na₂HPO₄, 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 35S-labeled antisense RNA complementary to vspα mRNA, which had been hydrolyzed to an average length of 250 nucleotides. The prehybridization treatments were as described (Meierowitz, 1987), and hybridization was carried out for 12 hr at 43°C in 30% 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 x 10^6 cpm/μL 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 μg/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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