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# Coregulation of Soybean Vegetative Storage Protein Gene Expression by Methyl Jasmonate and Soluble Sugars[1]

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## ABSTRACT

**The soybean vegetative storage protein genes *vspA* and *vspB* are highly expressed in developing leaves, stems, flowers, and pods as compared with roots, seeds, and mature leaves and stems. In this paper, we report that physiological levels of methyl jasmonate (MeJA) and soluble sugars synergistically stimulate accumulation of *vsp* mRNAs. Treatment of excised mature soybean (*Glycine max* Merr. cv Williams) leaves with 0.2 molar sucrose and 10 micromolar MeJA caused a large accumulation of *vsp* mRNAs, whereas little accumulation occurred when these compounds were supplied separately. In soybean cell suspension cultures, the synergistic effect of sucrose and MeJA on the accumulation of *vspB* mRNA was maximal at 58 millimolar sucrose and was observed with fructose or glucose substituted for sucrose. In dark-grown soybean seedlings, the highest levels of *vsp* mRNAs occurred in the hypocotyl hook, which also contained high levels of MeJA and soluble sugars. Lower levels of *vsp* mRNAs, MeJA, and soluble sugars were found in the cotyledons, roots, and nongrowing regions of the stem. Wounding of mature soybean leaves induced a large accumulation of *vsp* mRNAs when wounded plants were incubated in the light. Wounded plants kept in the dark or illuminated plants sprayed with dichlorophenyldimethylurea, an inhibitor of photosynthetic electron transport, showed a greatly reduced accumulation of *vsp* mRNAs. The time courses for the accumulation of *vsp* mRNAs induced by wounding or sucrose/MeJA treatment were similar. These results strongly suggest that *vsp* expression is coregulated by endogenous levels of MeJA (or jasmonic acid) and soluble carbohydrate during normal vegetative development and in wounded leaves.**

We previously investigated changes in protein and mRNA populations that occur when soybean seedlings are exposed to water deficit (3, 6, 7, 16, 17). These studies showed that a 28-kD protein accumulates in the hypocotyl cell wall fraction when soybean seedlings are transplanted to low water potential soil (3, 16). This protein and an immunologically related 31-kD protein are also abundant in soluble hypocotyl fractions (16) and localized in vacuoles (H.S. Mason, L.R. Griffing, and J.E. Mullet, unpublished data). Analysis of the cDNAs that encode the 28- and 31-kD proteins (16) revealed that they are identical with the soybean leaf VSP[2] first identified by Wittenbach (34) and whose cDNAs were subsequently sequenced by Staswick (25). The VSP are glycoproteins that accumulate in leaf paraveinal mesophyll and bundle sheath cells (10) and, in some situations, in epidermal cells (28). The 28- and 31-kD subunits, designated VSP-$\alpha$ and VSP-$\beta$, respectively, show 80% sequence identity but differ substantially in charge (16) and form homo- and heterodimers (24). Analysis of genomic clones indicated that the VSP are encoded by a small multigene family of at least three members (20). The genes *vspA* and *vspB* encode the subunits VSP-$\alpha$ and VSP-$\beta$, respectively.

The VSP accumulate in leaves before anthesis, decline during pod filling, and increase again after seed maturation (26). Removal of developing pods from soybean plants or petiole girdling to block transport from leaves cause the accumulation of the VSP and their mRNAs, as well as two other proteins, in leaves (26, 34). The modulation of *vsp* expression during mobilization and transport of leaf materials to developing reproductive sinks led to the suggestion that the VSP serve a temporary storage function (34). This idea is consistent with the accumulation of the VSP and *vsp* mRNAs in water-limited plants in which growth is inhibited (16, 17, 30). The VSP and their mRNAs are also abundant in developing leaves, stems, pods, and flowers but rare in roots, seeds, and mature stems and leaves (17, 27). This pattern of expression is consistent with the role of the VSP as a temporary sink for carbon and nitrogen in growing regions of the shoot.

We studied the expression of the soybean *vsp* with the hope of gaining insight into the molecular signals that control storage protein deposition during shoot development and in response to water deficit. One plant signal that is of particular importance for *vsp* gene expression is JA, a growth regulator that is probably derived from linolenic acid and has structural similarity to animal eicosanoids (1). JA and its methyl ester, MeJA, are widely distributed in plants (1, 18) and can induce changes in protein and mRNA populations when applied to plants (2, 8, 17, 19, 29). Very low concentrations (0.1–10 $\mu$M) of JA or MeJA induce the accumulation of VSP-$\beta$ (2) and *vspB* mRNA (17) in soybean cell cultures. These results indicate that JA/MeJA can modulate the expression of *vsp*.

In this paper, we identify soluble carbohydrate as a modulator of *vsp* expression. We report that the wound induction of *vsp* mRNAs is greatly enhanced in illuminated as compared with dark-treated plants, which points to a role for photosynthetic carbon in the regulation of wound-induced *vsp* expression. Furthermore, the distributions of MeJA, TSS, and *vsp* mRNAs in dark-grown soybean seedlings are well correlated,

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[2] Abbreviations: VSP, vegetative storage protein(s); JA, jasmonic acid; JA/MeJA, jasmonic acid and/or methyl jasmonate; MeJA, methyl jasmonate; TSS, total soluble sugars.

suggesting that *vsp* expression is coregulated *in vivo* by JA/MeJA and soluble carbohydrates.

## MATERIALS AND METHODS

### Materials

(±)MeJA was obtained from Bedoukian Research (Danbury, CT). Enzymes used for probe synthesis were from Gibco-BRL (Gaithersburg, MD). Unless otherwise noted, all other chemicals were obtained from Sigma (St. Louis, MO).

### Plant and Cell Culture Conditions

Soybean (*Glycine max* Merr. cv Williams 82) plants were grown in pots in a growth chamber with 18 h of light during the day (light intensity = 350 $\mu Em^{-2}\ s^{-1}$, 28°C days, 25°C nights) in Metromix 352 potting soil. Plants were watered as needed with half-strength Hoagland medium and grown until the middle leaflet of the sixth trifoliate leaf was about 2 cm long. Conditions for wounding and leaf excision for each treatment are described in the figure legends. Dark-grown soybean seedlings were cultivated and dissected into different regions as previously described (16). Soybean suspension cultures were obtained from Suzanne Rogers, Department of Horticultural Sciences, Texas A&M University, and grown photomixotrophically as described before (17). Changes in culture conditions for specific experiments are noted in the figure legends.

### mRNA Quantitation

Total nucleic acid was isolated, fractionated on formaldehyde gels, blotted to nylon membranes, and hybridized with antisense RNA probes as described previously (17). The probes specific for *vspA* and *vspB* mRNAs were as described before (17). β-Tubulin mRNA was detected using an antisense RNA made from plasmid pGE23, which encodes a soybean β-tubulin (7). The *cab* probe was generated from a 0.7 kilobase cDNA fragment from pea (4) by random primed labeling using a kit from Gibco-BRL according to the product literature. Hybridization conditions were as described for RNA probes (17) except that the temperature was 42°C and washing was in 0.2× standard sodium citrate at 50°C. Radioactivity in specific bands on the blots was quantified by scanning blots with a Betascope 603 blot analyzer (Betagen Corp.), and the data are presented in the figures as relative mRNA levels for each specific probe. The scale of the ordinate on the graphs showing mRNA levels is the same for *vspA* and *vspB* probes but differs for the *cab* and pGE23 probes. All experiments depicted in the figures were performed at least twice.

### Determination of TSS and MeJA

Soluble sugars were extracted from lyophilized plant tissues in 80% aqueous ethanol at 90°C and quantified as described previously (6), using glucose as a standard. To quantitate MeJA, we synthesized a heavy isotope-labeled MeJA for use as an internal standard by methylating JA with $^{13}CD_2N_2$. To obtain JA for use as a precursor in this reaction, MeJA was hydrolyzed with methanolic KOH, the resulting solution acidified, and the JA partitioned into ether. To convert the carboxylic acid of JA to its deuterated form, 0.25 g (1 mmol) of JA in ether (0.5 mL) was washed with $D_2O$ (0.5 mL) four times. $^{13}CD_2N_2$ (2.5 mmol) was generated from *N*-methyl-$^{13}$C-*N*-methyl-$D_3$ diazald (99% atom % $^{13}$C, 99.5 atom % D; Aldrich Chemical Co., Milwaukee, WI) using a mini-diazald apparatus according to the manufacturer's instructions (Aldrich). [$^{13}CD_3$]MeJA was synthesized from $D_2O$-washed JA in the presence of deuterated solvents as recommended by the manufacturer (Aldrich). GC-MS analysis of compounds was performed using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5970B mass selective detector. GC conditions were isothermal for 1 min at 80°C followed by temperature programming to 180°C at 25°C/min using a 25-m DB-1 (0.25 mm i.d., 1 μm phase thickness) column at a column pressure of 30 kPa with He as carrier. The retention time of MeJA was approximately 10 min. GC-MS-selected ion monitoring was performed by monitoring m/z 151, 156, 160, 177, 181, 193, 224, and 228. Dwell time for all ions was 100 ms. Sensitivity limits (m/z 224) were approximately 0.5 to 1 ng/μL. A calibration curve of various amounts of unlabeled and [$^{13}CD_3$]MeJA was used to estimate endogenous MeJA levels. Mature soybean hypocotyl tissue (6–30 g fresh weight) was harvested and frozen immediately in liquid $N_2$. Tissue was stored at −60°C until extracted. Plant samples, after addition of 0.875 to 3.5 μg [$^{13}CD_3$]MeJA, were homogenized in acetone. The acetone was removed by rotary evaporation and the aqueous phase (after acidification to pH 2.5) partitioned into ether. The ether phase was dried over anhydrous sodium sulfate and removed by rotary evaporation. The residue was dissolved in 89% hexane:10% ethyl acetate:1% acetic acid (v/v/v). MeJA was further purified by isocratic (89% hexane:10% ethyl acetate:1% acetic acid, v/v/v) silica gel (5 μm particle size, 4.5 × 250 mm) HPLC at a flow rate of 2 mL/min. Fractions corresponding to MeJA (3.0 min) were collected, dried, dissolved in a small volume of ethyl acetate (usually 20–100 μL), and analyzed by GC-MS-selected ion monitoring as described above. The recovery of [$^{13}CD_3$]MeJA ranged from 40 to 60%.

## RESULTS

### Accumulation of *vsp* mRNAs in Excised Mature Leaves Requires Both Sucrose and MeJA

In an earlier study, we observed that *vsp* mRNA levels increased when dark-grown soybean seedlings were illuminated (17). Because this increase could have been due to the activation of photosynthesis and the accumulation of photoassimilate, we tested whether the treatment of excised mature leaves with sucrose would modulate the levels of *vsp* mRNAs. Leaflets of fully expanded trifoliate leaf 1 (numbered acropetally) were used because, in contrast to young growing leaves (Fig. 1A, L6), *vsp* mRNAs are rare in mature leaves (Fig. 1A, L2; ref. 17). When leaflets were excised and floated on liquid media in the dark, treatment with 0.24 M sucrose or 10 μM MeJA alone had only small effects on *vsp* mRNA levels (Fig. 1A). In contrast, addition of both sucrose and MeJA dramatically increased *vspA* and *vspB* mRNA levels, which continued to accumulate throughout a 3-d period (Fig.

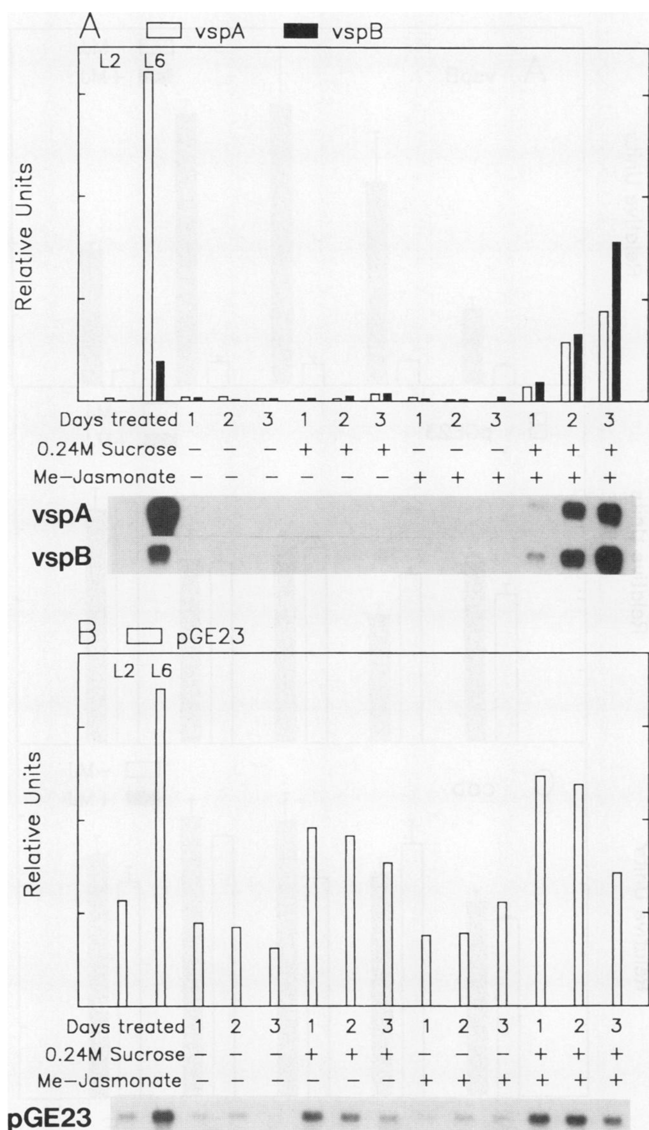


**Figure 1.** Induction of *vsp* mRNAs by sucrose and MeJA (Me-Jasmonate) in mature soybean leaf. Individual leaflets of the first trifoliate were excised, surface sterilized with 1% NaClO, washed extensively, and floated on solutions containing Murashige-Skoog salts and vitamins, with or without additions of 0.24 M sucrose and/or 10 $\mu$M MeJA for 1, 2, or 3 d at 25°C in the dark. Total nucleic acid was isolated and fractionated on formaldehyde gels (2.0 $\mu$g/lane). Radioactivity on the blots was quantified directly with a $\beta$-particle scanner, and the relative amounts were plotted. L2 and L6 are nucleic acid samples from intact trifoliate leaf 2 (mature) and leaf 6 (growing), respectively. A, Samples probed for *vspA* and *vspB* mRNAs; B, same samples probed for $\beta$-tubulin (pGE23) mRNA.

1A). For comparison, levels of an mRNA encoding a soybean $\beta$-tubulin (pGE23) were assayed using the same samples and are presented in Figure 1B. The levels of mRNA hybridizing with the pGE23 probe were low in untreated and excised control leaflets, which is consistent with earlier findings that this mRNA is abundant in growing tissues and less prevalent in nongrowing tissues (7). Addition of sucrose to the medium increased $\beta$-tubulin mRNA levels about twofold, but MeJA had little effect and the sucrose/MeJA synergism seen with *vsp* mRNAs was absent (Fig. 1B).

### Sucrose, Fructose, and Glucose, but Not Amino Acids, Stimulate *vsp* Expression

To examine the effect of sucrose on *vsp* expression more closely, we used a soybean suspension culture system. The cultures were grown under constant illumination with varying amounts of sugars present and stimulated with MeJA 3 d after passage. For reasons unknown, only *vspB* mRNA is induced in this culture (17). The effect of sucrose concentration on the ability of MeJA to induce *vspB* mRNA accumulation is shown in Figure 2A. Although sucrose alone had little effect, in conjunction with 10 $\mu$M MeJA, it stimulated the accumulation of *vspB* mRNA. The sucrose effect was maximal at a concentration of 58 mM, and similar *vspB* mRNA levels were seen at sucrose concentrations up to 145 mM (Fig. 2A). In contrast, the level of a $\beta$-tubulin mRNA (pGE23 probe) was increased by the addition of sucrose but was not further affected by MeJA (Fig. 2B). We also probed these RNA samples with a *cab* cDNA. The level of *cab* mRNA is increased in illuminated plants (4) but was modulated only to a small extent by the sucrose and MeJA treatments used here (Fig. 2C).

Fructose and glucose were as effective as sucrose in potentiating the induction of *vspB* mRNA by MeJA, as shown in Figure 3A. The sugars in the media were supplied as equivalent mass, because sucrose in these cultures is normally hydrolyzed very quickly by invertase (23). The monosaccharides had slightly higher *vspB* mRNA-inducing activity in conjunction with MeJA than did sucrose. Fructose and glucose were also effective in potentiating the MeJA induction of *vspB* mRNA in excised leaves (H.S. Mason, D.B. DeWald, R.A. Creelman, and J.E. Mullet, unpublished data). When the amides Gln and Asn were added at 5 mM each along with 116 mM sucrose, no effect on *vspB* mRNA level was seen, but the amides inhibited the MeJA induction of *vspB* mRNA by about 30% (Fig. 3A). Again, the levels of $\beta$-tubulin (pGE23, Fig. 3B) and *cab* (Fig. 3C) mRNAs were not altered by treatment with MeJA.

### Distribution of *vsp* mRNAs, MeJA, and Soluble Sugars in Soybean Seedlings

The levels of *vsp* mRNAs are high in the apical growing region of hypocotyls relative to the nongrowing regions of the stem and the roots of dark-grown soybean seedlings (17). If JA/MeJA and soluble carbohydrate concentrations coregulate the expression of *vsp*, then they should be positively correlated with *vsp* mRNA levels in different regions of the seedlings. Thus, we measured *vsp* mRNA, TSS, and MeJA levels in 3-

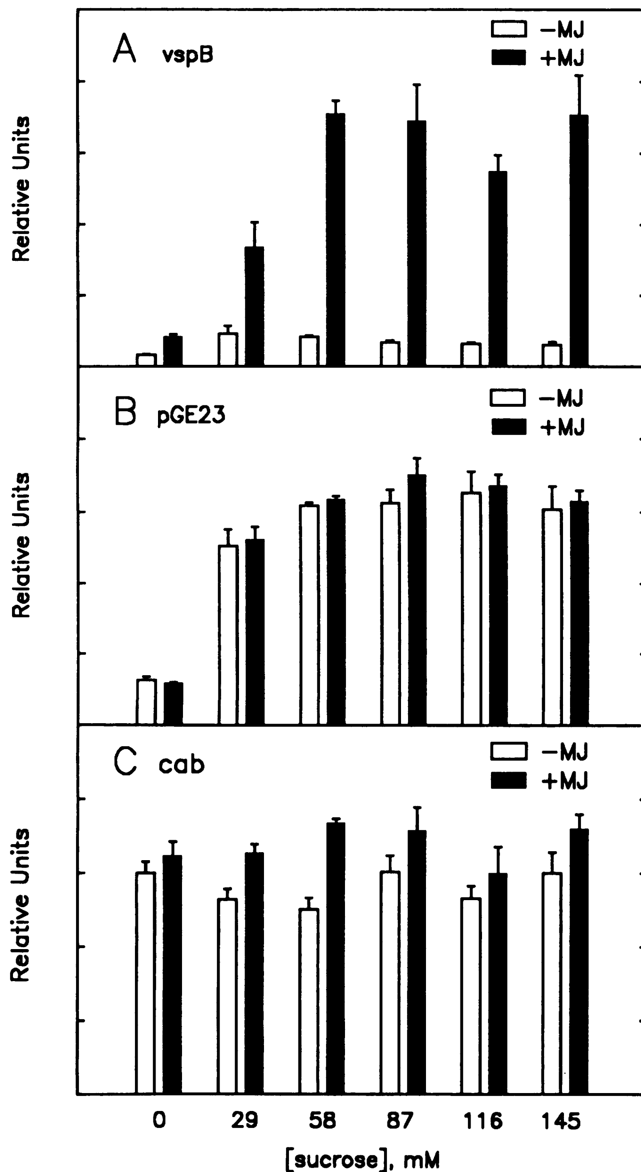


**Figure 2.** Sucrose concentration dependence for *vspB* mRNA induction by MeJA (MJ) in suspension-cultured soybean cells. A single suspension culture was subdivided and passed into media containing 0, 29, 58, 87, 116, or 145 mM sucrose. Three days after passage, a portion of each culture was harvested and the remaining portion made to 10 μM MeJA. The MeJA-stimulated cultures were harvested after 24 h treatment. Total nucleic acid was isolated, fractionated (13 μg/lane), and probed, and radioactivity on the blots was quantified as in Figure 1. Probes were (A) *vspB* antisense RNA, (B) pGE23 antisense RNA, and (C) *cab* random primed DNA. Column, mean (error bar, ± SD) of values obtained from three different cultures.

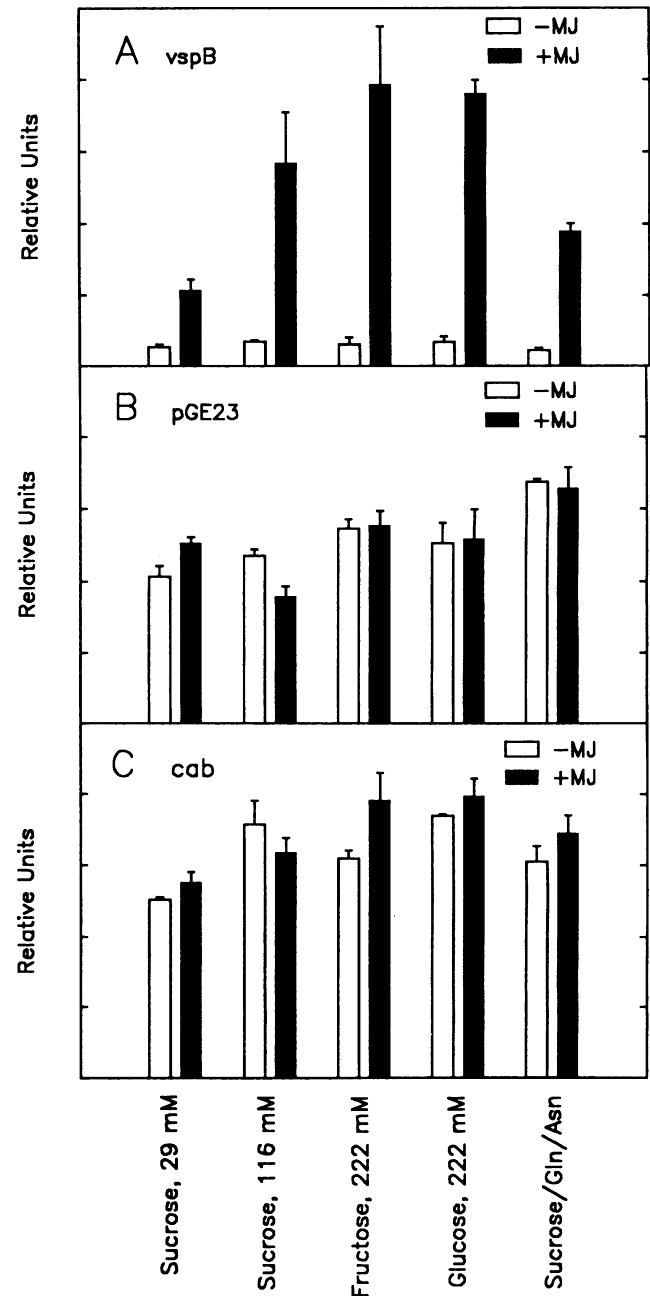


**Figure 3.** Substitution of fructose and glucose for sucrose in *vspB* mRNA induction by MeJA (MJ) in suspension-cultured soybean cells. Cultures were passed into media containing 29 mM sucrose, 116 mM sucrose, 222 mM fructose, 222 mM glucose, or 116 mM sucrose + 5 mM each of Gln and Asn. The cultures were treated with 10 μM MeJA as in Figure 3, and total nucleic acid was isolated, fractionated (13 μg/lane), and probed, and radioactivity on the blots was quantified as in Figure 1. Probes were (A) *vspB* antisense RNA, (B) pGE23 antisense RNA, and (C) *cab* random primed DNA. Column, mean (error bar, ± SD) of values obtained from three different cultures.

d-old dark-grown soybean seedlings, and the data are shown in Figure 4. TSS were highest in the hypocotyl hook and elongating regions, but substantial amounts occurred in the other tissues as well (Fig. 4A). Soluble sugars in dark-grown soybean seedlings occur mainly as fructose, glucose, and sucrose (in order of decreasing abundance, ref. 14). Sorbitol also occurs at a substantial level (about 10% of the total, ref. 14) but would not have been detected by the phenol-sulfuric acid method because it has a reduced carbonyl. The TSS data in Figure 4 are consistent with those of Kuo *et al.* (14), excluding the sorbitol data. Based on the TSS data and fresh to dry weight ratios, we estimate that the molar concentrations of glucose equivalents ranged from 20 mM in the root to 60 mM in the hypocotyl hook and elongating regions, assuming a uniform distribution among cell types and subcellular compartments.

MeJA levels were highest in the hypocotyl hook, which had about 2.5 times more than the hypocotyl elongating region on a fresh weight basis (Fig. 4A). Molar concentrations of MeJA calculated as above for TSS ranged from approximately 0.2 μM in the mature hypocotyl and root to 3.6 μM in the hypocotyl hook. The abundance of *vsp* mRNAs in the different seedling regions (Fig. 4B) correlated very well with the levels of MeJA in the same regions. The β-tubulin mRNA (pGE23 probe) was most abundant in the hypocotyl elongating and hook regions, followed by the mature hypocotyl and root (Fig. 4B). Thus, β-tubulin mRNA levels were highest in growing tissues, as previously observed (7).

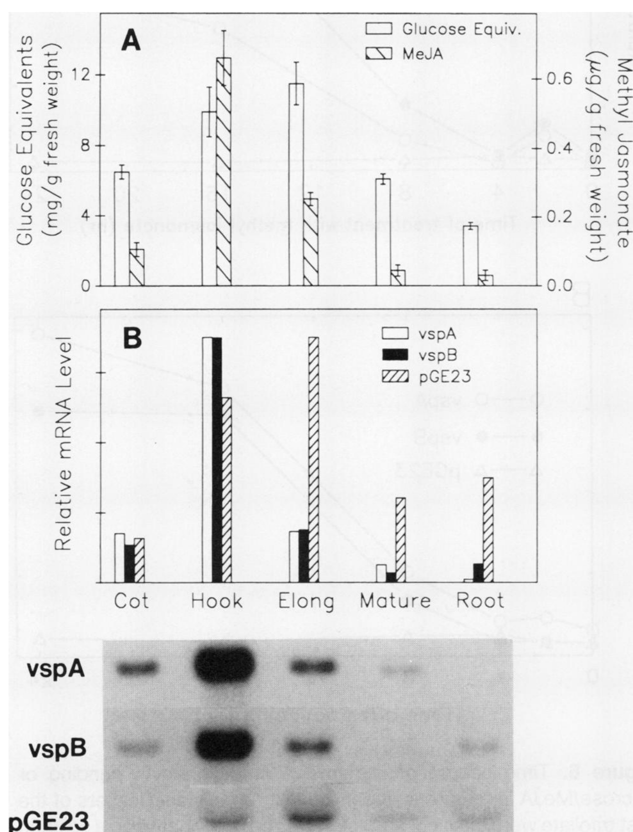


**Figure 4.** Distributions of TSS, MeJA, and *vsp* and pGE23 mRNAs in dark-grown soybean seedlings. Three-day-old dark-grown soybean seedlings were dissected to give cotyledons, hypocotyl hook, elongating and mature regions, and total root. A, TSS as glucose equivalents (columns, means [error bars, ± SD] of four samples) and MeJA (columns, means [error bars, ± SD] of three samples) in the different seedling regions. B, Total nucleic acid from the different seedling regions was fractionated (4 μg/lane) and probed for *vspA*, *vspB*, and pGE23 mRNAs, and radioactivity was quantified as in Figure 1. Columns, means of two determinations.

## *vsp* mRNA Levels Are Modulated by Wounding and Photosynthesis

It has been suggested that the induction of *vsp* mRNAs in wounded leaves is caused by the generation of JA/MeJA in the wounded tissues (17, 28, 29). The results in Figure 1 suggest that leaf *vsp* mRNA levels are coregulated by sucrose and MeJA. If this is correct, then the induction of *vsp* mRNAs in wounded leaves should be modulated by photosynthetic activity. To test this idea, we determined the effects of light and DCMU, an inhibitor of photosynthetic electron transport, on the induction of *vsp* mRNAs by wounding. Medial leaflets of the first trifoliate leaves were wounded, and RNA from these and unwounded lateral leaflets from the same plants was assayed for specific mRNAs.

Wounding induced a massive accumulation of *vsp* mRNAs in the medial leaflet and a substantial buildup in the unwounded lateral leaflet when the plant was incubated in the light after wounding (Fig. 5A, plant 2). Incubation of the wounded plant in the dark reduced the induction of *vsp* mRNAs about 20-fold (Fig. 5A, plant 1). Spraying wounded plants with DCMU in the light reduced the accumulation of *vsp* mRNAs about fivefold (Fig. 5A, plant 4). The slight induction seen in the dark was not affected by DCMU treatment (Fig. 5A, plant 3). Induction of *vsp* mRNAs in the unwounded lateral leaflet by wounding the medial leaflet indicates that a transportable wound signal can modulate *vsp* mRNA levels. For comparison, the levels of β-tubulin mRNA in the leaves described in Figure 5A were determined (Fig. 5B). This mRNA was about two- to threefold more abundant in illuminated wounded medial leaflets and unwounded lateral leaflets relative to dark- or DCMU-treated plants. Approximately the same degree of stimulation was observed in Figure 1 when excised leaves were treated with sucrose. This may indicate that β-tubulin mRNA levels are modulated to some extent by soluble carbohydrate availability.

The results in Figure 5 are consistent with the hypothesis that *vsp* expression is coregulated by wound-induced JA/MeJA and a product of photosynthesis. If this hypothesis is correct, then the time courses for induction of *vsp* mRNAs by wounding and MeJA/sucrose treatment may be similar. This experiment is shown in Figure 6. A small increase in *vsp* mRNAs was seen 2 h after initiation of treatment with sucrose/MeJA, but the main increase occurred between 4 and 16 h (Fig. 6A). A similar time course was observed with wounded, illuminated leaves (Fig. 6B). In contrast, mRNA hybridizing to the β-tubulin probe (pGE23) showed little variation in response to wounding or treatment with sucrose/MeJA (Fig. 6).

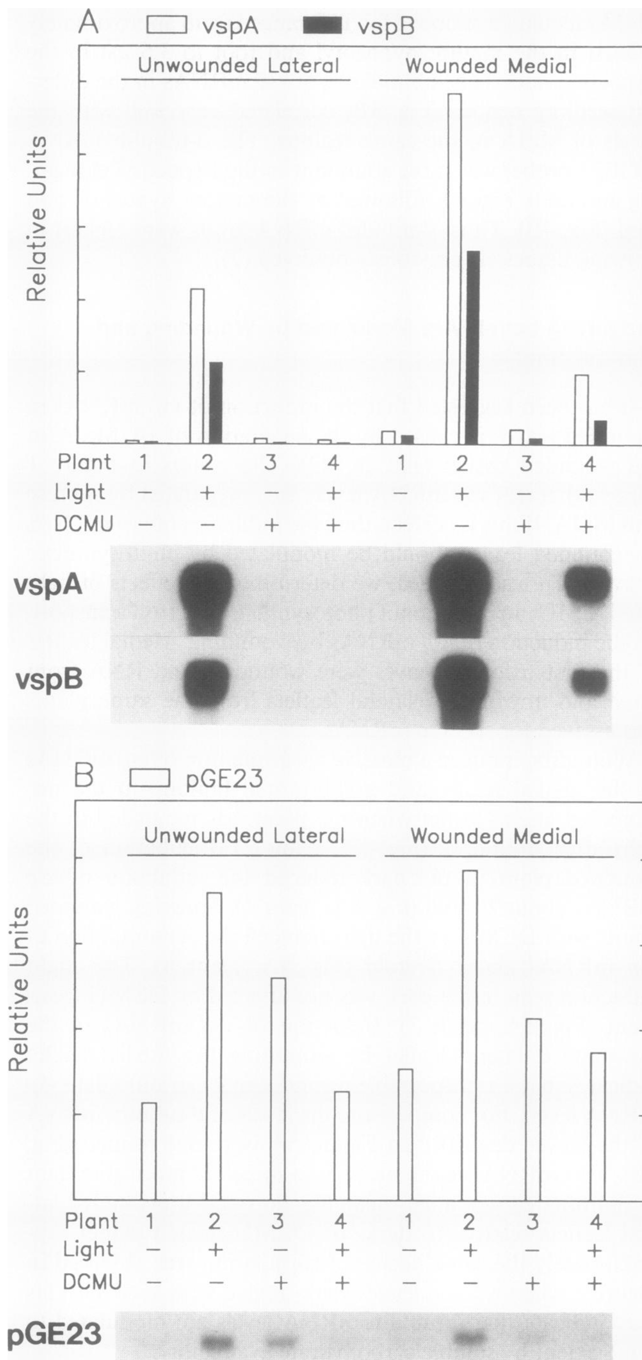


**Figure 5.** Wound induction of *vsp* mRNAs in mature soybean leaves. The distal ⅓ of the medial leaflet of the first trifoliate of four different plants was wounded by mashing 10 times with a hemostat. Plants 1 and 3 were incubated in the dark for 24 h, and plants 2 and 4 were incubated in continuous light for 24 h after wounding. The first trifoliate of plants 3 and 4 were sprayed four times during the 24-h period with a solution of 0.4 mM DCMU/0.05% Tween-20. After 24 h, the wounded medial leaflet and an unwounded lateral leaflet from the first trifoliate of each plant were harvested. Total nucleic acid was isolated, fractionated on formaldehyde gels (3.5 $\mu$g/lane), blotted to nylon membranes, and probed with antisense RNA probes specific for (A) *vspA* and *vspB* mRNAs, and (B) $\beta$-tubulin (pGE23).

## DISCUSSION

The soybean VSP were first designated as VSP because of their accumulation in leaves when pods were continuously removed from soybean plants (34). The accumulation of VSP under these conditions was thought to result from deposition of mobilized material in the transport pathway which connects senescing leaves and developing reproductive structures. This idea was further supported by the finding that VSP accumulate primarily in the vacuoles of leaf paraveinal mesophyll and bundle sheath cells (10). These cells provide a conduit for materials that are transported from palisade and

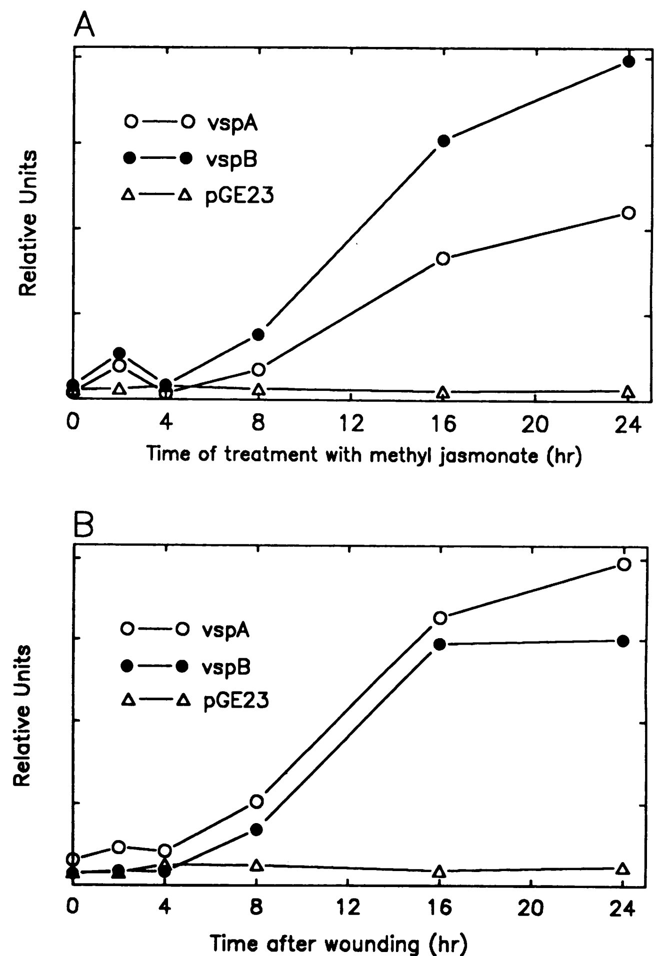


**Figure 6.** Time course of *vsp* mRNA induction by wounding or sucrose/MeJA in mature soybean leaves. A, Individual leaflets of the first trifoliate were excised, surface sterilized, and floated on a solution containing Murashige-Skoog salts and vitamins and 0.2 M sucrose in the dark for 24 h. Leaflets were then transferred to the same solution containing 10 $\mu$M MeJA and incubated for various times up to 24 h. Point, average of two replicate leaflets treated in the same way. B, Leaflets of trifoliate leaves 1 and 2 (experiment 1) or trifoliate leaf 1 only (experiment 2) on six plants were wounded as in Figure 2. At time points thereafter, wounded leaflets were collected, and total nucleic acids were isolated, fractionated (4 $\mu$g/lane), and probed as in Figure 1. Radioactivity was quantified as in Figure 1 and plotted against time after treatment with MeJA (A) or wounding (B). Point, average of experiments 1 and 2.

spongy mesophyll cells to phloem elements (9). More recently, it was found that the VSP and/or their mRNAs accumulate in young developing stems, leaves, and reproductive structures, and their levels decline when these tissues mature (17, 26, 27). Therefore, the VSP appear to serve a temporary storage function during the early phases of organ development and again during senescence when materials are mobilized from these structures. In the present paper, we have investigated the signals that modulate *vsp* expression with the long-term goal of understanding how protein storage in vegetative tissue is regulated in well-watered and water-deficient plants.

### Signals that Modulate *vsp* mRNA Levels

This study and others have demonstrated that JA/MeJA stimulates the accumulation of *vsp* mRNAs when soybean plants are sprayed or explants treated with 10 to 50 $\mu$M JA/MeJA (17, 28, 29). In soybean cell cultures, addition of 0.1 to 10 $\mu$M JA or MeJA results in VSP-$\beta$ protein and mRNA accumulation (2, 17, 28). We report here that MeJA was present in soybean seedling tissues at concentrations varying from about 0.2 $\mu$M in the root to 3.6 $\mu$M in the hypocotyl hook, assuming uniform distribution in the tissue. This indicates that the induction of *vspB* mRNA in cell cultures by MeJA occurs in a physiologically relevant concentration range, although we realize that compartmentation of MeJA could cause locally higher concentrations. Together, these data strongly implicate JA/MeJA as a modulator(s) of *vsp* expression in soybean.

We also identified soluble sugars as another group of compounds that modulate *vsp* expression. Addition of 0.24 M sucrose and 10 $\mu$M MeJA to excised leaves cultured in the dark greatly stimulated the accumulation of *vsp* mRNA, but neither compound alone caused a substantial increase (Fig. 1). The sucrose/MeJA synergism was also observed in soybean cell cultures, in which the MeJA-stimulated accumulation of *vspB* mRNA was dramatically increased by the addition of sucrose at concentrations up to 58 mM, whereas sucrose added without MeJA had no effect (Fig. 2). These results are consistent with recent data showing that VSP levels increase when soybean cell cultures are treated with sucrose and JA (2). In that work, the optimal sucrose level for VSP-$\beta$ accumulation was found to be 5.8 mM (2), whereas our data indicate that at least 58 mM sucrose was optimal for the accumulation of *vspB* mRNA. Differences in the cell culture conditions such as light intensity and $CO_2$ level may account for these observations.

The stimulation of MeJA-induced *vsp* mRNA accumulation was also observed with fructose or glucose. This is not surprising because sucrose is rapidly hydrolyzed in soybean cell cultures (23). The effect of sugars was probably not simply osmotic, because maximal stimulation occurred at a sucrose concentration of 58 mM (Fig. 2). This represents an osmotic potential of approximately −0.14 MPa, which is relatively small. By comparison, the osmotic potential of mature soybean leaf is approximately −0.8 MPa (H.S. Mason, D.B. DeWald, R.A. Creelman, and J.E. Mullet, unpublished data), which represents a sucrose concentration of 0.3 M. More recently, we found that treatment of cell cultures with PEG-8000 at a concentration that approximates the osmotic potential of 116 mM sucrose caused no stimulation of *vsp* mRNA accumulation, either with or without MeJA (H.S. Mason, D.B. DeWald, R.A. Creelman, and J.E. Mullet, unpublished results). Furthermore, the range of sugar concentrations that stimulated *vsp* mRNA accumulation is physiologically relevant. We estimated that the concentration of TSS was about 60 mM glucose equivalents in the seedling regions that showed high *vsp* mRNA levels. Other data indicate that total nonstructural carbohydrate levels in illuminated mature soybean leaves are approximately 2.5 to 4% of dry weight, which represents about 15 to 25 mM in glucose equivalents (5).

The soybean *vsp* join a group of plant genes that are modulated by soluble carbohydrates. Genes encoding the tuber storage proteins patatin and sporamin are induced in leaf explants by sucrose concentrations in the range of 0.1 to 0.3 M (12, 33). In contrast, the expression of maize photosynthetic genes (21) and *Shrunken* (sucrose synthase, ref. 15) are repressed by sucrose concentrations in the same range. The *vsp* and wound-inducible proteinase inhibitor II (13) genes are stimulated at lower levels of sucrose (fully induced at 30–60 mM sucrose). The basis of this difference in sensitivity to sucrose concentration awaits further investigation.

In addition to JA/MeJA and soluble sugars, the nitrogen status of soybean plants modulates *vsp* expression. A recent study showed that nonnodulated plants require at least 5 mM $NH_4NO_3$ in their nutrient solution to accumulate *vsp* mRNA, whereas *rbcS* transcripts accumulate at 1 mM $NH_4NO_3$ (29). This result may indicate that plants can restrict the expression of genes such as *vsp* to conditions in which nitrogen levels are sufficient to allow protein storage. In our experiments with light-grown plants and cell cultures, the nutrient solution contained at least 5 mM $NH_4NO_3$. Therefore, the effects of sugars and MeJA observed in this study occurred in plants that were not limited for nitrogen. Addition of Gln and Asn to soybean cell cultures (Fig. 3) and leaf explants (H.S. Mason, D.B. DeWald, R.A. Creelman, and J.E. Mullet, unpublished data) did not further stimulate *vsp* mRNA accumulation and inhibited the sucrose/MeJA stimulation somewhat.

The expression of *vsp* could be limited by either JA/MeJA or soluble sugar levels or both. We addressed this problem by assaying TSS, MeJA, and *vsp* mRNA levels in different parts of dark-grown soybean seedlings (Fig 4). The concentration of MeJA was calculated to be approximately 3.6 $\mu$M in the hypocotyl hook where *vsp* mRNA was most abundant and 0.2 $\mu$M in the root where *vsp* mRNA levels were lowest. TSS were highest (60 mM) in the hypocotyl hook and zone of cell elongation and 15 to 25 mM in the other tissues examined. Thus, *vsp* mRNA abundance was better correlated with MeJA than TSS level. For example, TSS did not differ greatly in the hook and elongating region, but the *vsp* mRNA level was fourfold lower and MeJA level was threefold lower in the elongating region. Earlier, we showed that MeJA treatment of dark-grown seedlings greatly increases the levels of *vsp* mRNAs in mature hypocotyls and roots (17), which suggests that the soluble sugar levels there are sufficient but that JA/MeJA is too low for accumulation of *vsp* mRNAs. In contrast, addition of MeJA to dark-treated mature leaves did not cause *vsp* mRNA accumulation unless sucrose was added, which indicates that both JA/MeJA and soluble sugars limit the expression of *vsp* in this tissue. Mild to moderate water deficit causes increases in the levels of *vsp* mRNAs in both dark-

grown seedlings and light-grown soybean plants (16, 17, 30). Soluble sugars also increase during water deficit in the hypocotyl zone of elongation of dark-grown seedlings (6), which suggests that sugars may mediate the effect on *vsp* expression.

Our data correlating *vsp* mRNA levels with TSS and MeJA levels in soybean seedlings do not prove, but suggest, a causal relationship. This analysis is limited by the lack of data concerning the local concentrations of these compounds in specific cell types and subcellular compartments. In addition, lack of a suitably labeled internal standard for JA at present limits our ability to quantify JA levels *in vivo*. However, these correlative data provide important information that is consistent with coregulation of *vsp* expression by soluble sugars and JA/MeJA. The nature of the soluble sugar effect is unknown, but two hypotheses are plausible. One is that a sugar (or a metabolite thereof) acts as a signal molecule that acts in concert with JA/MeJA to induce the expression of *vsp* genes. Another explanation is that JA/MeJA is the sole signal molecule for induction of *vsp* transcription, and energy sources in the form of carbon enhance this process. Thus, cells may have a way of expressing genes discriminately under conditions where energy is limiting (*i.e.* low ATP/ADP ratio). In an effort to distinguish between these two possibilities, we are presently analyzing *vspB* promoter regions in transgenic tobacco.

### Modulation of *vsp* mRNA Levels in Wounded Tissue

In an earlier study, we observed that *vsp* mRNA levels increased in wounded tissues, and we hypothesized that wounding increased the synthesis of JA/MeJA, which in turn stimulated the accumulation of *vsp* mRNAs (17, 29). We recently demonstrated that JA/MeJA levels increase rapidly in wounded soybean tissue (R.A. Creelman and J.E. Mullet, unpublished data) which is consistent with previous work showing that a precursor of JA, 12-oxo-phytodienoic acid, accumulates in wounded tissue (31). In addition, inhibitors of lipoxygenase were found to block the accumulation of *vsp* mRNAs in wounded soybean leaves (29), which is consistent with the lipoxygenase dependent synthesis of JA from linolenic acid (32). In our earlier experiments, the extent of wound-inducible *vsp* mRNA accumulation was variable. We now believe that this can be explained by variation in the soluble carbohydrate status of the wounded tissue.

In this paper, we show that the extent of *vsp* mRNA accumulation in wounded mature leaves was much greater when the leaves were illuminated. The influence of light could be largely reversed by DCMU, an inhibitor of photosynthetic electron transport (Fig. 5). These results suggest that the light-stimulated carbon fixation in leaves increases the wound-induced *vsp* mRNA accumulation. The levels of *vsp* mRNAs in wounded leaves reached a maximum between 12 and 24 h postwounding (Fig. 6). Similar induction kinetics are observed when soybean cell cultures are treated with MeJA in the presence of sufficient levels of sucrose (17). When the ability of light and soluble carbohydrate was recognized to stimulate *vsp* mRNA accumulation, it could be demonstrated that *vsp* mRNA levels increased in unwounded lateral leaflets adjacent to wounded medial leaflets (Fig. 5, ref. 28). This indicates that *vsp* can respond to a signal transmitted from the wounded leaflet. The nature of the wound signal is unknown but could be JA/MeJA.

The ability of *vsp* to respond to a transmissible wound signal is similar to that of the genes encoding proteinase inhibitors in potato and tomato. Like *vsp*, the proteinase inhibitor II gene exhibits sucrose-enhanced wound induction (13) and responsiveness to MeJA (8). Furthermore, like the VSP, proteinase inhibitor I is localized in vacuoles, is highly expressed in shoot growing zones, and is itself regarded as a reserve protein (22). Also, proteinase inhibitor I is normally absent from mature tomato leaves unless they are wounded and incubated in the light (11). Although the VSP and proteinase inhibitors share little sequence identity, it appears that both have been recruited to serve as temporary storage proteins. To date, we have been unable to demonstrate that the soybean VSP have proteinase inhibitor activity.

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