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Nitrogen and Methyl Jasmonate Induction of Soybean Vegetative Storage Protein Genes

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ABSTRACT

Vegetative storage protein (VSP) and VSP mRNA levels in soybean (Glycine max) leaves correlated with the amount of NH4NO3 provided to nonnodulated plants. The mRNA level declined as leaves matured, but high levels of N delayed the decline. This is consistent with the proposed role for VSP in the temporary storage of N. Wounding, petiole girdling, and treatment with methyljasmonate (MeJA) increased VSP mRNA in leaves 24 hours after treatment. The magnitude of the response depended on leaf age and N availability. N deficiency essentially eliminated the response to wounding and petiole girdling. MeJA was almost as effective in N-deficient plants as in those receiving abundant N. Inhibitors of lipoxygenase, the first enzyme in the jasmonic acid biosynthetic pathway, blocked induction by wounding and petiole girdling but not by MeJA. This supports a role for endogenous leaf jasmonic acid (or MeJA) in the regulation of VSP gene expression.

Soybean VSP2 is an abundant vacuolar glycoprotein which was first isolated from leaves by Wittenbach (30). VSP is thought to play an important role in the temporary storage of N because it accumulates abundantly in several immature organs. As these organs mature and begin exporting nutrients to other developing tissues, VSP is preferentially lost (for review see ref. 25). Several factors increase the expression of leaf VSP genes, including seed pod removal, blockage of leaf phloem export (23), wounding (15, 25), and water deficit (14). These responses also appear related to the need for N storage, but the molecular signals that regulate the VSP genes are unknown.

VSP consists of two related polypeptides which form heteroand homodimers (21). The amino acid sequences have been deduced from leaf (22) and hypocotyl (14) cDNAs. The VSP mRNAs are approximately 1.1 kilobases in size and the protein subunits have a molecular mass of approximately 25 kD, although the proteins differ slightly in relative mobility by SDS-PAGE. The high and low mobility subunits have been named VSPα and VSPβ, respectively, and their corresponding genes, vspA and vspB (15).

VSP accumulates in the vacuoles of bundle sheath and paraveinal mesophyll cells in leaves (9). The latter are considered an extension of the bundle sheath system and have been implicated in the temporary storage and redistribution of N during plant growth (9). VSP also accumulates in the upper epidermal cells of immature leaves (25). VSP mRNA has a similar distribution in seedling hypocotyls, where it is associated with epidermal and vascular tissues (15).

A novel plant compound, JA (3-oxo-2-(2'-cis-pentenyl)-cyclopentane-1-acetic acid), and its methyl ester (MeJA) were recently shown to induce the accumulation of VSP polypeptides in soybean leaves and in soybean suspension cell cultures (3). VSP mRNA is also increased by JA and MeJA (15, 25). JA may be an important regulator of plant gene expression, because it is widely distributed in plant species (13) and it induces the accumulation of specific leaf polypeptides in several species (7, 10).

The structural similarity between JA and animal prosta-glandins has been noted (29). Oxidation of linolenic acid by lipoxygenase is the first step in JA biosynthesis (27), and JA has been suggested to play a role in stress signaling in plants (2). JA is generally most abundant in developing organs (11, 28) as are the VSP gene products. However, it remains to be established whether endogenous JA regulates plant gene expression.

In this study we examined whether or not N availability influenced VSP gene expression in soybean leaves, because N storage appears to be an important function for VSP. The results are consistent with the hypothesis that N is first allocated to more essential proteins. VSP is synthesized according to the availability of excess N. We also investigated the role of endogenous JA in mediating VSP gene expression. Several inhibitors of the JA biosynthetic pathway decreased VSP induction by wounding and phloem blockage, but they did not block VSP induction by MeJA.

MATERIALS AND METHODS

Plant Material

Experiments on the effect of N availability were done with a nonnodulating soybean line (Glycine max L. Merr., strain T181 rj.). Seeds were germinated for 3 d on germination paper, and then two seedlings were transplanted to washed sand and vermiculite (1:1) in a 10-cm pot. Plants were grown in a controlled environment chamber for 16-h days (27°C day and 22°C night). Plants were watered without nutrients for the first week. They were then watered every other day during the second week, and daily for the final 2 weeks, with 50 mL

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2 Abbreviations: VSP, vegetative storage protein; JA, jasmonic acid; MeJA, methyljasmonate.
of nutrient solution (26) which contained various concentrations of NH4NO3. Nodulated plants (strain T180 Rj1) were grown under the same conditions in field soil without additional nutrients. Leaves were harvested 28 d after planting, a few days before anthesis. Leaves were stored at -80°C until analysis.

Leaves for the lipoxygenase inhibitor studies were from the cultivar "Hobbit," which was grown under the same temperature and light regimen in field soil. Leaves for explants were excised near the main stem. The petiole was immediately cut again under water 1 cm from the basal end. Explants were transferred to flasks or test tubes for the various treatments described. They were then cultured at 25°C under continuous light until harvest.

Lipoxygenase Inhibitor Treatment

Mature leaves from the second and third nodes were harvested 2 to 3 weeks after anthesis from nodulated plants grown in an environment chamber as described earlier. Explants were courted for 24 h as described above in 50 μM of one of the following: antipyrone (2,3-dimethyl-1-phenyl-3-pyrrolozin-5-one), ibuprofen (α-methyl-4-[2-methylpropyl]benzeneacetic acid), indoprofen (4-[1,3-dihydro-1-oxo-2H-isonoindol-2-yl]-α-methylbenzeneacetic acid), mafenamic acid (2-[2(3-dimethylphenylamino)benzolic acid], phenylbutazone (4-buty1-1,2-diphenyl-3,5-pyriodinedione), n-propyglycine (3,4,5-trihydroxybenzoic acid n-propyl ester), or salicyldroxyamic acid (N,2-dihydroxybenzamid). Ibuprofen was first dissolved in 10⁻⁴ volume of methanol and then added to the aqueous solution. Solutions were stirred overnight before use. The pH of solutions was maintained near neutrality with 0.2 mM potassium phosphate, pH 7. All inhibitors were from Sigma Chemical Co. MeJA was obtained from Bedoukian Research Inc., Danbury, CT.

Protein Analysis

Soluble protein extraction, SDS-PAGE, antisera production, immunoblotting, and protein quantitation were done as previously described (23).

RNA Isolation and Analysis

Total RNA was obtained from frozen powdered tissue by an acid guanidinium thiocyanate-phenol-chloroform extraction procedure (5). The pellet obtained following propanol precipitation was dissolved in a LiCl buffer and extracted as described by De Vries et al. (6). RNA was electrophoresed on agarose-formaldehyde gels and blotted to nitrocellulose as described by Fournet et al. (8). The RNA slot blot procedures and hybridizations were as described previously (23).

RESULTS

N Enhances VSP Gene Expression

The influence of N on VSP gene expression was studied in a nonnodulating soybean line that was grown with the levels of N indicated in Table I. Plants receiving 0 or 1 mM NH4NO3 were chlorotic and had only two fully expanded trifoliolate leaves 4 weeks after transplanting. All other plants had three fully expanded leaves. Leaves of plants supplied with 2 mM NH4NO3 were yellow-green. All others appeared dark green and similar to the control, a nodulated isoline grown in field soil. The amount of total protein extracted from leaves of each N treatment is indicated in Table I. The protein content of plants grown with 2 or 5 mM NH4NO3 was similar to the control. Based on these observations, plants receiving 0 and 1 mM NH4NO3 were judged to be deficient in N relative to the nodulated control. Above 40 mM NH4NO3, plants showed signs of N toxicity.

VSP accumulation in leaves is correlated with N availability (Fig. 1A). Plants supplied with 1 or 2 mM NH4NO3 accumulated VSP to about the same level as nodulated plants. This was previously shown to be approximately 3% of the soluble leaf protein at this stage of leaf development (23). VSP was increased in plants receiving more N. By comparison of band intensities with a dilution series of VSP, we estimated that the relative abundance of VSP was about 15-fold higher in plants receiving 40 mM NH4NO3 than in those receiving 2 mM. VSP was not detectable on this blot for plants receiving no added N. The higher mobility subunit (VSPα) was consistently more abundant than the other, although both were increased by N. Previous studies demonstrated that VSP accumulation is closely associated with VSP mRNA abundance. RNA blot hybridizations (Fig. 1B) were analyzed to determine whether N also increased the amount of VSP mRNA. The results correlated well with the protein data. Longer exposure of the film revealed faint bands for the plants receiving 0 and 1 mM NH4NO3, but the mRNA in these samples was about 50-fold less abundant than in plants receiving the highest amount of N (see also Fig. 2). Treatment with 40 mM NH4NO3 resulted in VSP mRNA accumulation to at least 30-fold greater than in nodulated plants.

To assure that the VSP mRNA differences were not due to a variation in the efficiency of mRNA extraction among N treatments, the same RNA samples were also hybridized with six other leaf cDNA clones. These clones were isolated at random from the cDNA library from which the VSP clones were obtained (22). A representative result for one of these, a ribulose-1,5-bisphosphate carboxylase small subunit cDNA, is shown in Figure 1B, inset. The relative abundance of all six messages was unchanged by the availability of N.

<table>
<thead>
<tr>
<th>Added N</th>
<th>Protein* mg/g fresh wt</th>
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<tbody>
<tr>
<td>mM NH4NO3</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.5 ± 2.2</td>
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<tr>
<td>1</td>
<td>15.7 ± 3.3</td>
</tr>
<tr>
<td>2</td>
<td>20.1 ± 2.3</td>
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<tr>
<td>5</td>
<td>30.9 ± 5.2</td>
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<tr>
<td>10</td>
<td>33.7 ± 4.8</td>
</tr>
<tr>
<td>20</td>
<td>30.3 ± 5.8</td>
</tr>
<tr>
<td>40</td>
<td>37.6 ± 2.8</td>
</tr>
<tr>
<td>Control*</td>
<td>24.5 ± 5.6</td>
</tr>
</tbody>
</table>

* The value given is the mean ± SD of four extractions. Control was a nodulated plant as described in "Materials and Methods."
leaves (two fully expanded) and the others had six (three fully expanded).

Even the youngest leaves (stage 1) from N-deficient plants contained less VSP mRNA than the oldest leaves (stage 6) of plants receiving the abundant N. The intermediate level of N resulted in a decreasing gradient of expression over a range of 100-fold from youngest to oldest leaf. This is consistent with previous results (23). In contrast, plants supplied with 30 mM NH₄NO₃ retained high levels of mRNA even in the most mature leaves. These contained only four- to eightfold less than the youngest leaves. These results demonstrated that although youngest leaves had the highest levels of VSP mRNA for all N treatments, the availability of N dramatically influenced the absolute level of accumulation at all stages of leaf development. We also examined whether or not the VSP genes could be induced in a given leaf after restoring N to deficient plants. Plants were first grown to the four-leaf stage with 1 mM NH₄NO₃ and then were grown with a daily supply of 50 mL of 30 mM NH₄NO₃. Regreening was visually detectable 4 to 6 d after restoring N. Leaves were harvested at the fourth node from the bottom of the plant. Deficient plants responded to the increased availability of N by increasing VSP mRNA (Fig. 2B). A slight increase was observed within 2 d and mRNA accumulation continued for several days. This experiment demonstrated that, even as leaves continued

SDS-PAGE analysis of the steady-state protein levels among these leaf samples also indicated that the relative abundance of most proteins did not vary (not shown). Similarly, the in vitro translation products derived from plants receiving 1 and 20 mM NH₄NO₃ were essentially the same (not shown). Within the detection limits of these experiments, the results indicate that the large increase in VSP gene expression caused by N is quite specific to these genes. However, total protein levels are lower in deficient plants. The increase of VSP gene expression is also specific for N, because increased levels of potassium, phosphate, or sulfur had no effect (not shown).

N Availability Modulates Developmental Gene Expression

Direct comparison of VSP mRNA levels between N treatments is confounded by the fact that the VSP genes are developmentally regulated. N-deficient plants developed slower and leaves were smaller than those on plants grown with sufficient N. To examine the effect of development and N nutrition, leaves at different developmental stages were examined. Plants were grown with 1.5, or 30 mM NH₄NO₃, which provided levels of N that were deficient, intermediate, and abundant, respectively. The relative amount of VSP mRNA in leaves of different developmental stages from these plants is shown in Figure 2A. Deficient plants had only four

Figure 1. Effect of N on VSP and VSP mRNA abundance. A, Total leaf protein (4 μg/lane) was probed with VSP antisera following electrophoretic transfer to nitrocellulose. Numbers above lanes indicate the concentration of NH₄NO₃ provided to these nonnodulated plants during growth. The control (lane C) was from a nodulated plant grown to the same age. B, Total RNA (5 μg/lane) from the same leaf samples shown directly above was electrophoresed in a formaldehyde-agarose gel. The blot transfer was hybridized with an equimolar mixture of the vspa and vspb cDNA inserts. The inset shows a hybridization to the corresponding samples with a ribulose-1,5-bisphosphate carboxylase small subunit probe.

Figure 2. Influence of N availability on the VSP developmental pattern of expression. A, Total RNA (1 μg) was vacuum blotted onto nitrocellulose and hybridized with VSP cDNA inserts. Plants were grown as in Figure 1 under the N regimens indicated. Length of leaves at the different stages of development were: stage 1, 0.5 to 1.5 cm; stage 2, 1.5 to 4.0 cm; stage 3, 4.0 to 7.0 cm; stages 4 to 6; >7.0 cm (fully expanded). A twofold dilution series for the stage 1 leaves from plants grown with 30 mM NH₄NO₃ is shown as a standard. B, RNA gel blot was prepared as described in Figure 1. N was restored to deficient plants and leaves were harvested after plants were grown with a daily supply of 50 mL 30 mM NH₄NO₃ for 0, 2, 4, 6, or 8 d.

NITROGEN AND JASMONIC ACID REGULATION OF VSP GENES

The stage 2 leaves to more than 200-fold for the stage 6 leaves grown with 5 mM NH₄NO₃. Placing leaf explants in water rather than MeJA for 24 h had essentially no effect on VSP mRNA relative to the respective leaves harvested immediately from plants (control).

In contrast, leaves from the N-deficient plants were only weakly induced (<eighthfold) 24 h after wounding or petiole girdling. However, MeJA resulted in a >200-fold increase in VSP mRNA. Extending the time after treatment to 72 h did not increase the magnitude of the response to any of these treatments (not shown).

To test whether mineral N or nitrogenous organic compounds could directly increase the level of VSP mRNA, explants were also incubated for up to 3 d with 0.5 mM NH₄NO₃, 0.5 mM glutamine plus 0.5 mM Asn, or 0.5 mM allantoin plus 0.5 mM allantoic acid. None of these altered VSP mRNA levels (not shown). Similarly, supplementing soybean suspension cell cultures (provided by J. Widholm) with these nitrogenous compounds did not increase VSP mRNA (not shown), although MeJA was effective (25).

Lipoxygenase Inhibitors Block VSP Gene Induction by Wounding and Petiole Girdling

Circumstantial evidence suggests that endogenous JA and/or its methyl ester may be involved in regulating VSP gene expression. To test this more directly, we examined whether VSP gene induction could be blocked by inhibitors of lipoxygenase, the first enzyme in the JA biosynthetic pathway. The effect of a 24-h pretreatment of leaves with various compounds previously shown to inhibit soybean seed lipoxygenase (17, 20) is summarized in Figure 4. Five inhibitors essentially eliminated the induction by petiole girdling. Two others (me-

to mature, VSP gene expression in them was increased by added N.

N Availability Influences VSP Gene Induction by Other Stimuli

Several diverse stimuli have been shown to induce VSP mRNA accumulation in leaves. We investigated the extent to which N availability influenced the increase in VSP mRNA in response to leaf wounding, petiole girdling, and the uptake of MeJA by leaf explants. Treatments were carried out for 24 h because earlier experiments showed that maximal induction occurred at this time. We also wanted to avoid diurnal effects which could result from harvesting at intervals other than 24 h. VSP mRNA is increased in leaves at night (23). We also found that 10 μM MeJA effectively increased VSP mRNA when assimilated by leaf explants but 1 μM did not (not shown). To assure a strong induction in the present experiments, we used 50 μM MeJA.

Leaves from all developmental stages of the plants supplied with 5 and 30 mM NH₄NO₃ were inducible to about the same absolute level by all treatments after 24 h (Fig. 3). Longer exposure of the X-ray film and comparison with standards indicated that the increases ranged from about twofold for

**Figure 3.** Influence of N on VSP induction by wounding, petiole girdling, and MeJA. Leaves were wounded by crushing the distal third of the leaflets with a hemostat, and the wounded portion was later harvested. Petioles were girdled with steam as described earlier (23). Leaf explants were placed in water or 50 mM MeJA and then returned to the growth incubator. All leaves were harvested 24 h after treatment. The controls are untreated leaves harvested at the time other leaves were treated.

**Figure 4.** Inhibition of VSP gene induction by lipoxygenase inhibitors. Leaf explants were allowed to take up a 50 μM solution of the indicated inhibitors or H₂O for 24 h. Petioles were then heat girdled and RNA was isolated after an additional 24 h in inhibitor solution. Control tissue was treated with inhibitors for 48 h without girdling.
fenamic acid and antipyrene) decreased the induction relative to incubation in water alone. The effect on wound-inducible and MeJA-inducible expression was also examined for the four most effective inhibitors. The response to wounding was also blocked by pretreatment with inhibitors (Fig. 5). However, VSP mRNA levels were still fully elevated by MeJA. These observations are consistent with the hypothesis that wounding and petiole girdling increase VSP gene expression at least partially via de novo synthesis of JA and/or MeJA.

**DISCUSSION**

The results demonstrate that VSP gene expression in soybean leaves is dependent on the availability of N. This is consistent with VSP's presumed function in temporary N storage. The low level of accumulation when N is scarce would permit more essential proteins to be synthesized first. When N is abundant, the excess could be temporarily stored and then released for reuse as needed. Similarly, VSP mRNA (and protein) increases in mature N-exporting leaves if leaf phloem export is blocked or the sinks (seed pods) for exported nutrients are removed (23). Under these conditions, the level of soluble N increases in leaves (12, 16). The storage of excess N in the form of protein may be a way to prevent accumulation of soluble N to toxic levels.

High expression in immature organs may also increase the sink strength of these organs for N. The accumulated N would then be readily available as these organs continue to develop, such as during leaf expansion and seed pod elongation (24). The N may also be exported to other sinks, such as new meristems and developing seeds.

Earlier studies revealed that VSP mRNA in a given leaf decreased as the leaf matured (23). Our present results demonstrate that N modulates the developmental pattern of VSP expression. VSP mRNA accumulation in even the youngest leaves of deficient plants was relatively low, and RNA disappeared early in leaf development. In contrast, high levels of N almost eliminated the normal decline in VSP mRNA in developing leaves. One explanation for this is that plants that have access to abundant N require less remobilization from their mature organs. Hence, N continues to be stored in mature leaves. Conversely, deficient plants may remobilize N from leaves early in their development and thus accumulate little VSP. This is consistent with the hypothesis that VSP genes are expressed according to the need to store N when it is available in excess.

Restoring N to 4-week-old deficient plants increased leaf VSP mRNA within 2 d, and the level continued to increase for another 6 d. This result parallels the situation for the pea seed storage protein legumin, which is greatly decreased under sulfur deficiency due to decreased legumin mRNA levels (4). Legumin mRNA increases for several days after restoring sulfur to deficient pea plants.

The results from the N experiments suggested that mineral N or important N-transport compounds, such as amino acids and urea, might act as a signal for VSP gene expression. However, NH$_4$NO$_3$ as well as amino acids and urea had no effect on VSP mRNA accumulation in leaf explants. Similarly, no induction by these compounds was detected in soybean suspension cell cultures, whereas MeJA had a dramatic and rapid effect (15, 25). Although we did not directly test for the assimilation of these compounds in the explant system, it seems likely that they were assimilated. We conclude that plant N nutrition dramatically influences VSP gene expression, but N does not appear to be directly involved in signaling the VSP genes.

That soluble leaf N does not directly signal N storage needs may be important for normal N metabolism. The breakdown of proteins and other macromolecules for their mobilization from exporting organs could result in the accumulation of soluble N awaiting export. If this triggered synthesis of VSP, a futile cycle could result.

Our results confirmed and extended previous findings that showed that leaf wounding and petiole girdling induced VSP mRNA accumulation (15, 23, 25). Moderate levels of N were a prerequisite for induction by these stimuli. In contrast, MeJA induced the accumulation of VSP mRNA to nearly the same absolute level in both deficient and healthy plants. We did not directly look for differences in the expression of the two VSP genes. Although both VSP subunits were increased by N, VSP$_\alpha$ accumulated to higher levels under all N treatments. This subunit is also more abundant throughout normal development, but depodding elevates both subunits to about the same level (23, 24). Recent work demonstrated that vspA mRNA was also somewhat more abundant than vspB mRNA after water deficit, wounding, or MeJA treatment (15). Although differences in the timing of induction have not been carefully examined, both VSP genes appear to be regulated similarly in leaves. In contrast, MeJA had little or no effect on vspA mRNA in suspension cells and in imbibed seedling axles, but it increased vspB mRNA (15).

Both JA and MeJA induce VSP protein and mRNA in leaves and suspension cell cultures (3, 15, 25). We tested only the methyl ester in this study because it was more effective than the acid at increasing VSP mRNA in the explant system.
Stimulating VSP gene expression by MeJA was found to be more reproducible if MeJA was assimilated through the vascular system of explants rather than spraying it on leaves (PE Staswick, unpublished results).

JA (and/or MeJA) is an attractive candidate for an endogenous signal that is involved in regulating the VSP genes. This study and others have clearly shown that exogenously applied JA increases VSP mRNA levels in soybean leaves, hypocotyls, and suspension cells. JA levels also correlate with VSP expression; both are highest in young tissues (11, 28). However, the degree of correlation with VSP gene expression needs further study. Another wound-inducible vacuolar leaf protein, potato protease inhibitor, was also recently found to be induced by MeJA (7).

This study provides new evidence that endogenous JA may regulate the VSP genes. All seven lipoxygenase inhibitors tested had some ability to block the induction due to girdling leaf petioles. For two of these (antipyrene and mfenamic acid), the inhibition was only weak. It should be noted that most of these compounds have low solubility in water, and we do not know how effectively they were taken up by the explant system. All four of the other inhibitors tested (n-propyisalicylhydroxamic acid) also blocked induction by wounding but not by exogenously applied MeJA. These results support a role for endogenous JA in regulating the VSP genes.

We cannot rule out the possibility that the inhibitors acted by a mechanism other than the inhibition of lipoxygenase, although the variety of compounds tested suggests otherwise. Salicylhydroxamic acid and n-propyisalicylhydroxamic acid are chelators of divalent cations and they inhibit the alternative respiratory pathway (19). Although the inhibitors we used are diverse in their structures (Fig. 6), there are similarities among them. It will be important to establish whether treatments such as seed pod removal, excess N, petiole girdling, and wounding increase endogenous JA and whether the lipoxygenase inhibitors block this putative increase.

If lipoxygenase and JA are involved in signaling plant stress and VSP gene expression, then it will be important to determine what regulates lipoxygenase activity and/or gene expression. Although soybean seed lipoxygenase has been well studied, little is known about the lipoxygenase(s) of vegetative tissues, which apparently differs from its seed counterpart (18). It has been suggested that another enzyme, 12-oxophytodienoic acid reductase, may be the rate-limiting step in JA biosynthesis, because its activity is low relative to lipoxygenase (29).

VSP gene induction by changes in endogenous JA levels may not require modulation of the activity of lipoxygenase or of other enzymes of the JA biosynthetic pathway. There is limited evidence that JA is transported via the phloem (1). If this is so, disruption of leaf phloem export could increase leaf JA levels in mature leaves without a net increase in JA biosynthesis. In this model, JA would be a sensitive detector of the import-export status of an organ. Additionally, stresses such as wounding could release fatty acids from cell membranes, perhaps through the action of phospholipases (2). Linolenic acid, the precursor for JA biosynthesis, undergoes oxidation by lipoxygenase. Thus, some plant stresses may increase JA biosynthesis by increasing substrate availability.

In summary, soybean has a sensitive mechanism to detect and store excess N in the form of VSP, N does not appear to directly induce the VSP genes. Instead, the endogenous JA level may be used to sense the import-export status of organs and regulate VSP gene expression accordingly. The gene promoter elements that mediate JA-inducible expression, as well as the JA signal transduction pathway (e.g., receptors?), are important new topics in plant biology which are worthy of further study.

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LITERATURE CITED