Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots

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This study presents the effects of methyl jasmonate (MeJA) on growth, N uptake, N partitioning, and N storage in taproots of non-nodulated alfalfa (cv. Lodi). When compared to untreated plants, addition of 100 \(^\mu\)M MeJA to the nutrient solution for 14 days reduced total growth and modified biomass partitioning between shoots and roots in favor of taproots and lateral roots. MeJA decreased N uptake (after 7 days) and increased N partitioning towards roots after 14 days. This preferential N partitioning to roots was accompanied by increased N storage in taproots as soluble proteins. Compared to total soluble proteins, VSP accumulation occurred earlier (7 days), and was greater (2-fold increase) in plants treated with 100 \(\mu\)M MeJA. Steady-state transcript levels for two VSPs (32 and 57 kDa) also increased markedly (about 4-fold) in roots of plants treated with 100 \(\mu\)M MeJA. This suggests that MeJA could act directly (transcriptional regulation) or indirectly (via the changes of N partitioning among alfalfa organs) on N storage as soluble proteins and in particular, VSPs. Because the deduced amino acid sequence of the 32 kDa VSP clone reveals high homology with Class III chitinases, we propose that the 32 kDa VSP may have a role in pathogen defense, in addition to its function as a storage protein.

Introduction

Many herbaceous and woody species are regularly exposed to critical growth phases during development, such as initiation of spring growth, grain filling, or regrowth after defoliation. These events often result in plants being confronted with a transient limitation of C and N resources (Chapin et al. 1990). In perennial forage legumes such as alfalfa (*Medicago sativa* L.), photosynthetic activity as well as N uptake or symbiotic N\(_2\) fixation (Volenc et al. 1996) are severely curtailed during early regrowth after defoliation. Post-clipping shoot regrowth requires the mobilization of C and N resources previously stored in roots (lateral roots and taproot) and crowns (Volenc et al. 1996). Studies using \(^{13}\)C and \(^{15}\)N labelling have shown that C is mainly used to support root and crown respiration (Ta et al. 1990, Avice et al. 1996b), while endogenous N pools (mainly represented by taproot soluble proteins and amino acids) were largely used to provide shoots with their N needs during herbage regrowth (Hendershot and Volenc 1993, Avice et al. 1996a, Barber et al. 1996). Four soluble proteins [15 19, 32 and 57 (β-amylase) kDa] have been identified in alfalfa taproots (Hendershot and Volenc 1993, Avice et al. 1996a, Cunningham and Volenc 1996, Gana et al. 1998). According to the criteria given by Cyr and Bewley (1990) and Staswick (1994), these proteins possess features consistent with being vegetative storage proteins (VSP). These particular polypeptides, mainly localized in vacuoles of parenchyma cells of wood rays and bark

**Abbreviations** — IRMS, Isotope Ratio Mass Spectrometer; JIP, jasmonate-induced protein; MeJA, methyl jasmonate; N, Nitrogen; PBS, phosphate buffer saline; PGT, phosphate gelatin Tween buffer; PNPP, p-nitrophenyl phosphate; PR protein, pathogenesis-related protein; PVDF, polyvinylidine difluoride.
of alfalfa taproots (Avice et al. 1996a), can represent up to 40% of the total soluble proteins, and show preferential mobilization/re-accumulation after defoliation and during initial shoot growth in spring.

Because of their important role during specific stages of plant development, the regulation of VSP accumulation has been investigated in poplar (Populus deltoides Bartr. ex Marsh), soybean (Glycine max L. Merr.), Arabidopsis thaliana, potato (Solanum tuberosum L.), chicory (Cichorium intybus L.), alfalfa (for review see Ourry et al. 2001) and oilseed rape (Brassica napus L.) (Rossato et al. 2002). VSP storage is modulated by changes in source-sink relationships for N within the plant. Modifications in N partitioning that led to VSP accumulation were induced by reducing shoot growth in poplar (Zhu and Coleman 2001), removal of pods in soybean (Tranbarger et al. 1991, Staswick 1994), or elimination of buds in potato (Paiva et al. 1983), soybean (Stephenson et al. 1998) and poplar (Coleman et al. 1993). Numerous studies also have shown that environmental signals such as daylength, temperature, light, N availability, drought, and wounding, influence VSP accumulation (Coleman et al. 1993, Van Cleve and Apel 1993, Staswick 1994, Noquet et al. 2001).

Furthermore, it appears that these environmental signals are relayed by endogenous signals within the plant. Recent studies in poplar (Beardmore et al. 2000, Zhu and Coleman 1990, Anderson 1991, Staswick 1994) and *A. thaliana* (Berger et al. 1995) clearly demonstrate that jasmonic acid, auxin, and gibberellins modulated VSP accumulation in storage tissues. Among these hormones, jasmonic acid, and its derivative methyl ester, methyl jasmonate (MeJA), which are naturally found in high concentrations in flowers and reproductive tissues (Staswick 1994, Creelman and Mullet 1997), rapidly enhance the vsp gene expression in soybean (Mason and Mullet 1990, Anderson 1991, Staswick 1994) and *A. thaliana* (Berger et al. 1995). Recently, Beardmore et al. (2000) also showed that 20 µM airborne MeJA induced bark storage protein (BSP) accumulation and expression of its corresponding genes in leaves and stems of young poplar (*Populus nigra* Muench × *Populus maximowiczii* A. Henry) plants. This MeJA effect on VSP accumulation was also recently reported in oilseed rape where it causes the accumulation of a 23-kDa VSP in taproots (Rossato et al. 2002).

MeJA, which is synthesized from linolenic acid in response to environmental or developmental signals like flowering, fruit ripening, wounding, pathogen attacks, tuberization, senescence or leaf abscission (Sembdner and Parthier 1993, Creelman and Mullet 1997, Koda 1997), was identified in several Leguminoseae species such as *Vicia faba*, *Pisum sativum*, *Phaseolus vulgaris* and *Lupinus albus* (Meyer et al. 1984). Preliminary works reported by Noquet et al. (2001) have shown that inclusion of MeJA (100 µM) during 35 days in the nutrient solution of non-nodulated alfalfa (cv. Europe) induced large modifications in N source-sink relationships within the plant that led to preferential N allocation towards roots and significantly increased taproot VSP concentrations. Nevertheless, little is known about the timing and dose–response effects of MeJA on growth and particularly, on the partitioning of N storage in alfalfa taproots.

Our objective was to investigate the impact of exposure time (7, 14 and 21 days) and dosage (1, 10, 100, 1000 µM) of exogenous MeJA on the growth and the N partitioning of non-nodulated alfalfa (cv. Lodi). The MeJA effects were studied at the physiological (biomass production and partitioning, N uptake, N source–sink relationships), biochemical (nitrate, amino acids, soluble proteins, VSP accumulation) and gene expression levels (VSP gene expression). This study reveals that the 32 kDa VSP gene (accession no. AF330579) is induced by MeJA, is a storage protein homologous to Class III chitinases, and possesses in vitro chitinolytic activity, suggesting that the 32 kDa VSP functions both in N storage and pathogenesis-related protein.

**Materials and methods**

**Plant material and culture**

Alfalfa seeds (*M. sativa* L., cv. Lodi) were sown on a synthetic substrate (Oasis growing pinpot, Agrimedia, France) on 4 August 1999 for the experiment with 100 µM MeJA for 7, 14 and 21 days, and on 21 October 1999 for the experiment with different concentrations of MeJA for 7 days. After 15 days, when the primary trifoliate leaves appeared, seedlings were transplanted to plastic pots filled with perlite and irrigated three times per week with 300 ml of a complete nutrient solution. This solution contained, in mM, 0.4 KH₂PO₄, 1 K₂SO₄, 3 CaCl₂, 0.5 MgSO₄, 0.15 K₂HPO₄, 0.2 Fe-Na EDTA; and in µM, 14 H₃BO₃, 5 MnSO₄, 3 ZnSO₄, 0.7 CuSO₄, 0.7 (NH₄)₆Mo₇O₂₄, and 0.1 CoCl₂ (Noquet et al. 2001). Nitrogen was supplied at 2 mM NH₄NO₃ to prevent nodule formation. Plants were grown under greenhouse conditions with a thermoperiod of 20°C (day) and 18°C (night) and a photoperiod of 16 h (day) and 8 h (night). After 4 months, plants were defoliated 6 cm above crown level and transferred to a continuously aerated nutrient solution in 8-l plastic containers. The composition of nutrient solution was identical to the above-described solution (except for NH₄NO₃, which was supplied at 1 mM) and was renewed every 3 days to prevent pH variation and NH₄NO₃ deficiency.

**Application of methyl-jasmonate**

Previous results (Avice et al. 1996a) have shown that the regrowth of alfalfa after defoliation was characterized by two different phases: during the first 2 weeks following shoot removal, there was an extensive mobilization of taproot organic N reserves (especially VSPs); thereafter, soluble proteins and VSPs were slowly re-accumulated. With the goal to study the effect of MeJA (Sigma Aldrich, Saint-Quentin Fallavier, France) on N/VSP re-accumulation, this phytohormone was applied after...
15 days of regrowth (Day 0) when the re-accumulation of proteins began. At Day 0, plants were supplied 100 \( \mu M \) MeJA for 7, 14 and 21 days, or different concentrations of MeJA (1, 10, 100 or 1000 \( \mu M \)) for 7 days. MeJA was directly supplied in the nutrient solution (renewed every 3 days) and plants grown without MeJA were considered as control plants. During all experiments, light was supplemented with sodium high-pressure lamps (phytoclaude 400W) supplying approximately 400 \( \mu \)moles photons m\(^{-2}\) s\(^{-1}\) 15 cm above crown with a photoperiod of 16 h (day) and 8 h (night). Throughout both experiments, the N source\(^{(15}\text{NH}_4\text{NO}_3, 1\text{mM})\) was labelled with 2.5 atom percentage \( ^{15}\text{N} \) excess to allow N analysis and to study N partitioning within the plant.

### Tissue sampling

Plants were sampled initially (Day 0) and after 7, 14 or 21 days of treatment. Harvested plants were separated into lateral roots, taproots, remaining leaves and stems in the crown (i.e. leaves and stems present on Day 0), and regrowing leaves and stems. FW of each sample was determined, and taproot samples were immediately frozen in liquid N\(_2\) and stored at \(-80^\circ\text{C}\). After freeze-drying, taproot samples were ground and kept at \(-20^\circ\text{C}\) for further N, protein, and VSP quantifications. Shoot tissues were dried at 70\(^\circ\text{C}\) for 72 h, ground to a fine powder, and stored under vacuum with desiccant for N analysis.

### Calculation of N partitioning in plant

The experiment (100 \( \mu M \) MeJA for 7, 14 and 21 days) was conducted with nutrient solution containing \( ^{15}\text{NH}_4^{15}\text{NH}_3 \) (with 2.5 atom\%\(^{15}\text{N} \) excess). The N content and \( ^{15}\text{N} \) abundance in all organs were measured in continuous flow using a C/N analyser linked to an isotope ratio mass spectrometer (IRMS, Roboprep CN and mass spectrometer, PDZ Europa Scientific, Crewe, UK). Natural \( ^{15}\text{N} \) abundance (0.3663\% \( \pm 0.0004 \)) of atmospheric N\(_2\) was used as reference for \( ^{15}\text{N} \) analysis. When N was taken up by the plant, it was labelled and the measurement of the \( ^{15}\text{N} \) abundance in the different organs could be used to determine the allocation of N derived from uptake during the time of experiment as we described previously (Avice et al. 1996b, Noquet et al. 2001).

Thus, nitrogen derived from current N uptake in a given organ (\( N_{\text{Up}} \)) was calculated as follows:

\[
N_{\text{Up}} = N_T \times (E(\%) / E_s(\%))
\]

where \( N_T \) is total nitrogen in organ (mg plant\(^{-1}\)), \( E(\%) \) is atom percentage \( ^{15}\text{N} \) excess in a given organ, and \( E_s(\%) \) is nutrient solution atom percentage \( ^{15}\text{N} \) excess (2.5\%).

Results are given as relative partitioning of N uptake (% of total N uptake) for each date of treatment (7, 14 and 21 days).

### Analysis of taproot N pools

Extraction of the different taproot N pools was performed according to the procedure previously described by Barber et al. (1996). Briefly, 300 mg of ground freeze-dried taproot were extracted twice with 5 ml of 100 \( \mu M \) sodium phosphate buffer (pH 7.0). Tissue suspensions were vortexed four times for 1 min at 5-min intervals and centrifuged at 3200 \( g \) for 20 min (4\(^\circ\text{C}\)). The resulting pellet represented the buffer-insoluble N fraction. The buffer-soluble protein N fraction was obtained from 3 ml of the resulting supernatants using the sodium deoxycholate-trichloroacetic acid (TCA) protocol described by Peterson (1983). After centrifugation (10 000 g, 10 min, 4\(^\circ\text{C}\)), the pellet represented the buffer-soluble protein-N fraction, while the supernatant was defined as the low molecular weight N fraction (consisting mainly of peptides, amino acids, and nitrate). N concentrations in the different taproot N fractions were determined using an isotope ratio mass spectrometer (Roboprep CN and mass spectrometer). Additionally, the nitrate concentration of the root extract was determined by the sulfanilamide method after reduction to nitrite using a continuous-flow autoanalyzer (Bran + Luebbe, Norderstedt, Germany). The pool of amino acids and peptides is deduced by subtraction of nitrate fraction from the low molecular weight N fraction.

### Extraction and analysis of taproot soluble proteins and VSPs

Protein analysis was adapted from Cunningham and Volene (1996). Proteins were extracted by suspending 300 mg of ground, freeze-dried taproot with 5 ml of extraction buffer (100 \( \mu M \) sodium phosphate, 10 \( \mu M \) \( \beta \)-mercaptoethanol, pH 7) at 4\(^\circ\text{C}\). After centrifugation (3200 \( g \), 4\(^\circ\text{C}\) for 20 min), the pellet was re-extracted in 5 ml of extraction buffer and the resulting supernatants were pooled. An aliquot of this extract was used for soluble protein measurement using protein dye-binding assay (Bradford 1976), VSP quantification by ELISA method, or VSP analysis by SDS-PAGE and Western blot as previously described by Noquet et al. (2001).

### Chitin-binding assays and chitinase activity

The protocol described by Gijzen et al. (2001) was used for chitin-binding assays on enriched VSP samples. VSPs were specifically extracted by suspending 1 g of lyophilized ground taproot powder in 10 ml of 50% (v/v) methanol as previously described by Barber et al. (1996). After centrifugation and protein precipitation of the resulting supernatant by sodium deoxycholate-TCA protocol (Peterson 1983), the protein pellet containing mainly VSPs was resuspended in 3 ml of protein extraction buffer (100 \( \mu M \) sodium phosphate, pH 7). One ml of this enriched-VSP suspension was added to approximately 100 mg chitin beads (New England Biolabs, Beverly, USA) and gently agitated for 1 h at 4\(^\circ\text{C}\). The chitin beads were pelleted (5000 \( g \), 5 min, 4\(^\circ\text{C}\)) and washed extensively with 2 ml of protein extraction buffer (Gijzen et al. 2001). Finally, the chitin beads extract or the enriched-VSP extract was prepared for analysis by
SDS-PAGE, by immunodetection or byzymogram to assay for chitinase activity. SDS-PAGE with Coomassie Brilliant Blue staining and immunodetection of 32 kDa VSP after Western blotting were performed as previously described by Noquet et al. (2001). For chitinase activity assays, protein samples (chitin beads or enriched VSP extract) were analysed on 15% SDS-polyacrylamide gel containing 0.01% (w/v) glycol chitin according to the method of Trudel and Asselin (1989). After SDS-PAGE, gels were washed with 1% (v/v) Triton X-100 in 100 mM sodium acetate buffer pH 5.0 at 37°C to re-nature proteins. Gels were visualized under UV light following staining with Calcofluor white M2R (Sigma, Aldrich) and lytic zones that correspond to chitinase activity were identified as non-fluorescent dark bands. Positive assay for chitinase activity was also performed with an extract of chitin from *Streptomyces griseus* (Sigma) (data not shown).

**Analysis of the 32 kDa VSP and β-amylase transcript levels**

Total RNA was extracted according to the procedure of Gana et al. (1998) adapted by Noquet et al. (2001). One gram of ground, freeze-dried taproot was placed in a tube containing 0.4 g of polyvinylpolypyrolidone (PVPP) and 4 ml of water-saturated phenol (65°C) and incubated at 65°C in a water bath. When samples thawed, 4 ml of RNA-extraction buffer (0.2 M sodium acetate, pH 5.2, 0.01 M EDTA, 1% SDS) was added to each tube, followed by incubation at 65°C for 15 min with 30 s of vortexing every 2 min. After 15 min incubation at 25°C, 4 ml of chloroform were added, and the tubes were shaken at room temperature for 10 min. Agitation of tubes, centrifugation, and recovery of the RNA pellet were conducted as described by Ougham and Davis (1990).

For Northern hybridization analysis, 5 µg of total RNA were separated on 1.5% agarose formaldehyde gels and transferred to nylon membranes (Gene Screen, NEN Life Science products, Boston, USA) using 10× SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7). The membranes were prehybridized (7% SDS, 0.25 M Na2HPO4, 2 mM EDTA, 0.2 mg ml−1 heparin, 0.1 mg ml−1 DNA calf thymus) for 2 h at 55°C with slow shaking and then hybridized with a 32P-labelled pMSBA1 β-amylase insert (GenBank accession AFO26217, Gana et al. 1998), and cross-linked onto the membranes by UV treatment (Bio-Rad, Ivry-sur-Seine, France). The 32P-labelled β-amylase probe was stripped from the membrane before rehybridization with a 32P-labelled 32 kDa insert (GenBank accession no. AF530579). The 32 kDa VSP and β-amylase inserts in the pBluescript SK (−) vector were isolated by digestion with *Xho*I and *Pst*I (32 kDa VSP) or *Hin*dIII (β-amylase) using an extraction kit (Quiagen, Gmbh, Hilden, Germany) and labelled with [32P]dCTP using random priming (Feinberg and Vogelstein 1983) with NE blot (New England Biolabs). Finally, in order to correct for RNA loading differences, the 32P-labelled 32 kDa probe was stripped from the membrane before rehybridization with a 32P-labelled pea 18S ribosomal probe. Details about conditions of hybridization were presented previously by Noquet et al. (2001). After each hybridization, membranes were exposed to phosphor screen and signals intensities were quantified using a Phosphor Imager (Packard Cyclone storage phosphor system, Packard Instruments Company, Niles, IL, USA). After each stripping, it was verified that the probe was completely removed using the Phosphor Imager. Following each quantification, membranes were exposed to radiographic film at −80°C and developed as described by the manufacturer (Eastman Kodak Company, New York, NY, USA).

**Statistical analysis**

The experiments were performed with 3 replicates (each replicate containing 4 plants). Results represented the mean ± SE for n = 3. The effect of MeJA concentrations was compared using t-test (Statview Student software, Abacus Concepts, Berkeley, USA).

**Results**

**Shoot and root biomass, shoot/root ratio**

The effects of 100 µM MeJA on biomass production was assessed after 7, 14 and 21 days of regrowth (Fig. 1). Addition of MeJA strongly reduced the total growth after 21 days and especially shoot dry matter production. Biomass partitioning between shoot and root tissues was strongly modified in favour of roots causing the shoot/ root ratio to decline from 2.6 (control plants) to 2.0 (MeJA treated plants). After 21 days, the total biomass...
of MeJA-treated plants was 33% lower than for untreated plants (Fig. 1).

N uptake and N partitioning in the whole plant
Total N uptake per plant (Fig. 2) and whole-plant N partitioning (Fig. 3) were studied using $^{15}$N labelling of MeJA-treated and control plants. By Day 14, MeJA induced a large decrease in N uptake per plant ($P < 0.05$; Fig. 2). After 21 days, N uptake was 2-fold less in MeJA-treated plants than in control plants. Throughout the experiment, MeJA strongly modified N partitioning between shoot and root organs (Fig. 3). After 14 days of treatment, MeJA reduced the allocation of N to regrowing shoots (Fig. 3A). N acquired by MeJA-treated plants was preferentially partitioned to roots (both taproots and lateral roots; Fig. 3C, D). After 14 days of treatment with MeJA, 67% of the exogenous N was distributed to the lateral roots, whereas only 51% lateral root N allocation was observed for control plants (Fig. 3D). During the same period, taproots accumulated 14% of the exogenous N in MeJA-treated plants compared to 11.5% in taproots of control plants (Fig. 3C). This difference in N partitioning to taproots of MeJA-treated and control plants also was observed on Day 21. These results showed that the addition of 100 μM MeJA in the nutrient solution induced a significant redistribution of exogenous N towards roots. From these results, we could expect this to alter the partitioning of N among root N pools leading to greater N storage as amino acids and/or soluble proteins in the taproot, and particularly as VSP.

N storage pools in taproots
The N concentration in taproots increased slightly between the Day 0 (22.6 mg N g$^{-1}$ DW) and Day 21 (24.6 mg N g$^{-1}$ DW). When compared to control plants, total N concentration in taproots was not significantly affected by MeJA treatment at any sampling (Table 1). Analysis of taproot N pools on Day 7 (Table 1) showed that the low molecular weight N fraction (nitrate, amino acids and peptides) and soluble protein N were not altered by MeJA treatment. However, MeJA reduced taproot nitrate concentrations after 14 days of treatment (Table 1). By Day 21, nitrate concentration in taproots of MeJA-treated plants was about 42% less than that of control plants (Table 1). By Day 14, the concentration of amino acids and peptides was increased upon MeJA
Day 0 22.65 ± 0.22 2.61 ± 0.29 2.49 ± 0.47 2.83 ± 0.34
Day 7 (+ MeJA) 21.13 ± 0.71 6.64 ± 1.27 2.75 ± 0.32 0.73 ± 0.05
Day 7 (control) 21.76 ± 0.86 6.43 ± 2.86 2.94 ± 1.27 0.75 ± 0.16
Day 14 (+ MeJA) 23.36 ± 1.02 8.88 ± 1.45 4.17 ± 0.64 0.51 ± 0.04
Day 14 (control) 25.01 ± 1.96 5.12 ± 1.97 3.60 ± 0.64 1.18 ± 0.15
Day 21 (+ MeJA) 24.66 ± 0.98 9.02 ± 1.52 3.18 ± 0.12 1.02 ± 0.13
Day 21 (control) 24.56 ± 0.40 5.17 ± 0.65 1.93 ± 0.40 2.43 ± 0.29

Table 1. Total N, soluble protein N, low molecular weight N pool (amino acids + peptides), and nitrate contents of alfalfa taproots after 7, 14 and 21 days of treatment with (100 μM) or without (control) MeJA. Methyl jasmonate application on Day 0 began 15 days after shoot removal in order to coincide with the onset of root N accumulation. Results are expressed as mg of N in a given fraction per g of taproot DW (mg N g⁻¹ DW) and are given as the mean ± se for n = 3. For soluble N pools, the results are also expressed as the percentage of total N (values in brackets).

Soluble proteins and VSP concentrations in taproots

The effect of MeJA application on soluble protein and VSP (32, 19, and 15 kDa) concentrations in taproots is shown in Fig. 4. On Day 14, soluble protein concentration was 45% greater for MeJA-treated than for control plants (Fig. 4A). ELISA quantification showed that total VSP concentration (sum of the 32, 19, and 15 kDa polypeptide signals) in taproots of control plants did not significantly change during the experiment (Fig. 4B). However, MeJA treatment doubled taproot VSP concentration by Day 7 (Fig. 4B). Thereafter, the VSP concentration continued to increase, reaching 27 mg g⁻¹ DW on Day 21 (Fig. 4B), and accounted for approximately 64% of the total soluble protein in taproots at this time. The analysis of Western blotting obtained after immunodetection with polyclonal antibodies anti-32 kDa indicated that the VSP of 32 kDa was strongly induced by MeJA in comparison with control plant (Fig. 4C). However, the abundance of 32 kDa progressively increased with the duration of treatment in control plants. This accumulation in control plants was expected because alfalfa plants were in the VSP re-accumulation phase of development. After 14 and 21 days of MeJA treatment, a protein with an estimated molecular mass of 30 kDa that cross-reacts with the anti-32 kDa antibodies was visible (Fig. 4C). The nature of this polypeptide is not known, but it has been previously observed using immunodetection in a previous work (Avice et al. 1997) when the 32 kDa VSP was highly accumulated in taproot of alfalfa grown under field conditions. These observations suggest that the 30 kDa polypeptide could be a precursor of the 32 kDa VSP.

Expression of 32 kDa VSP and β-amylase transcripts

On Day 7, steady-state transcript levels for the 32 and 57 kDa (β-amylase) VSPs of MeJA-treated taproots were significantly greater (average 4.25-fold increase) when compared to control plants (Fig. 5). The abundance of both transcripts remained high on Day 14 of MeJA treatment, averaging 2.7 and 4.8 greater in MeJA-treated roots for the 32 kDa and β-amylase transcripts, respectively. On Day 21, the relative abundance of the 32 kDa VSP and β-amylase transcripts in taproots of MeJA-treated plants decreased slightly, but were still about 2-fold higher than control plants (Fig. 5). The expression of both transcripts also progressively increased during the experiment in taproot of control plants, indicating that the alfalfa was in the period of re-accumulation of its protein reserves as previously reported in several studies (Volenc et al. 1996). Previous work indicates that β-amylase also may function as a VSP in roots of alfalfa (Gana et al. 1998).

Dose-effect of MeJA on soluble proteins and VSP concentrations

Because our results described above showed that 100 μM MeJA induced accumulation of VSPs on Day 7, we investigated, in a second experiment, the dose-effect of MeJA (1, 10, 100 and 1000 μM) on soluble protein and VSP (15, 19, and 32 kDa proteins) accumulation after 7 days of treatment (Fig. 6). The soluble protein concentration in taproots was not significantly affected by the...
addition of increasing concentrations of MeJA. However, taproot VSP concentrations increased by about 2-fold for 10 and 100 μM MeJA and 3-fold for 1000 μM MeJA, respectively, in comparison with control plants. Because soluble protein concentration remained constant (approximately 17.5 mg g⁻¹ DW on average), the proportion of VSP in the total soluble protein pool increased with the MeJA concentration from 50.4% (100 μM of MeJA) to 84.7% (1000 μM of MeJA) of the total soluble proteins (Fig. 6). However, plants treated with the highest MeJA concentrations showed visible symptoms of senescence, including chlorosis of the leaves after 7 days of MeJA treatment.

Characterization of 32 kDa VSP gene and chitinase activity assay

The clone of 32 kDa HMW VSP was registered in the GenBank database as accession no. AF530579. The complete nucleotide sequence of 32 kDa HMW was 1319 bp long. The open reading frame encodes a protein of 328 amino acid residues with the N-terminal 18 amino acids predicted to be a signal peptide (Fig. 7) using SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/). The mature peptide (verified with N-terminal sequencing) has a predicted pI of 4.9 and calculated molecular mass of 34.107 kDa (Fig. 7). Alignments of the deduced amino acid sequence of alfalfa 32 kDa VSP from several plant species revealed a 75% to 68% identity with Class III chitinase of *Sesbania rostrata* (accession no. Z48671).
and *Glycine max* (accession no. AB000097), respectively (Fig. 7).

By means of SDS-PAGE, Western blot analysis and the zymogram analysis using chitin as a substrate (Fig. 8), we tested the functional properties of the 32 kDa VSP. SDS-PAGE with Coomassie Brilliant Blue staining showed that a single protein band of approximately 32 kDa could be highly purified from an enriched VSP extract (Fig. 8A) or crude protein extract (data not shown) by binding to chitin affinity beads. Immunoblot analysis (Fig. 8B) revealed that anti-32 kDa VSP antibodies cross-reacted with the 32 kDa and 19 kDa VSP of the enriched VSP extract and also with the 32 kDa chitin-binding protein purified with chitin beads. Using zymogram analysis, the chitinase activity assay indicated that both 32 kDa VSP and the 32-kDa chitin-bound protein possessed chitinolytic activity (Fig. 8C). These results suggested that the 32 kDa VSP can bind and hydrolyse chitin.

**Discussion**

The addition of methyl-jasmonate (MeJA) at 100 μM to the nutrient solution reduced alfalfa growth on Day 21 of treatment and strongly modified the biomass partitioning between shoot and root tissues in favour of the root system (taproot and lateral roots). These results agree with previous studies that reported MeJA effects on plant growth, such as the inhibition of the seedling growth (Dathe et al. 1981) and the promotion of leaf senescence (Ueda and Kato 1980). In addition, application of MeJA to roots led to a significant \( P < 0.01 \) reduction in N uptake (2-fold decrease) even though there were no significant differences between total N in taproot of control and MeJA treated plants (Table 1). This was due to the MeJA-induced changes in the N partitioning among the different organs that resulted in preferential N allocation to the root system on Days 14 and 21 of treatment. These results are consistent with observations recently reported in oilseed rape (Rossato et al. 2002) where nitrate uptake was reduced by 90% after 8 days of MeJA treatment and N was preferentially translocated to roots at the expense of aerial tissues. Similar results have been reported for poplar (Beardmore et al. 2000) and for soybean (Franceschi and Grimes 1991) where it has been suggested that MeJA regulates N partitioning either by altering source-sink relationships with respect to N utilization, or by increasing the amino acid N storage rate. Additionally, the accumulation of N in root tissues in response to MeJA treatment may result from the inhibition of N transport from roots to shoots as amino acids.

Our results show that MeJA application alters source-sink relationships and partitioning of N among various N pools in alfalfa taproots. On Day 21 of treatment, there was a significant increase in N accumulation detected as soluble proteins, VSP N pools and minor accumulation of amino acids and peptides, while the nitrate and buffer-insoluble N pools decreased. These data suggest that the activities of enzymes involved in the reduction and in the assimilation of mineral N are not reduced by a treatment with MeJA. Compared with control plants, VSP accumulation in alfalfa taproots occurred rapidly (by Day 7) and was earlier than total soluble protein accumulation (by Day 14). Additionally,
we showed that the induction of VSP accumulation by MeJA was observed before a significant change in biomass or N flow occurred. MeJA treatments of varying concentrations after Day 7 did not appear to affect the total soluble protein pool, while VSP concentrations increased at least 2-fold when MeJA was applied at concentrations of 10 μM or higher. This suggests that MeJA could specifically stimulate VSP accumulation even at low MeJA concentrations. Moreover, the analysis of steady-state transcript levels for two alfalfa VSPs (32 and 57 kDa) indicates that 100 μM MeJA treatment induces transcript accumulation for both VSP mRNAs on Day 7 and beyond. This stimulation of gene expression in response to MeJA suggests that the promoters of both VSP genes may have the same cis-regulatory elements. Therefore, as previously reported for soybean (Mason and Mullet 1990, Franceschi and Grimes 1991, Staswick et al. 1991), MeJA may act at two levels in regulating VSP accumulation in alfalfa taproots: a direct action on VSP gene expression (after 7 days), and an indirect action on N partitioning (after 14 days) by altering source-sink relationships leading to preferential N allocation to storage organs. Similarly, studies conducted by Beardmore et al. (2000) in poplar revealed that topical application of MeJA induced a rapid response (72 h) increase in bsp gene expression, followed by BSP accumulation. Long-term topical application of MeJA resulted in organ-specific alterations in protein accumulation, N concentration, and BSP accumulation, as well as, preferential partitioning of biomass to stems without changing poplar relative growth rate.

Among the factors able to modulate VSP accumulation, it was shown that leaf wounding stimulated VSP expression and synthesis in soybean (Mason and Mullet 1990), A. thaliana (Berger et al. 1995) and poplar (Davis et al. 1993). Furthermore, jasmonates have also been implicated in the signal transduction pathway mediating systemic defense responses (Farmer and Ryan 1990, Baldwin et al. 1997). Jasmonates stimulated numerous genes involved in insect and pathogen resistance such as protease inhibitors in tomato (Lycopersicon esculentum), Farmer and Ryan (1990), potato (Hildmann et al. 1992) and soybean (Creelman and Mullet 1997). Farmer et al. (1992) demonstrated that the expression of an alfalfa trypsin inhibitor gene was highly induced after leaves were wounded or exposed to MeJA. Creelman and Mullet (1997) suggested that, in response to wound signals generated by insect or pathogen attacks, linolenic acid is released into the cytoplasm from plant cell membrane lipids and is rapidly converted to JA or MeJA, which can be translocated through the phloem to other tissues and serves as a signal to regulate the expression of proteinase inhibitor and VSP genes. In response to pathogen attack, plants have also developed different strategies such as the induction of several pathogenesis-related (PR) proteins like β-1-3 glucanases (Maunich et al. 1984), lysozymes, and chitinases (Boller 1988, Collinge et al. 1993). Many plant chitinases, which hydrolyse the β-1-4 linkage of chitin in fungal cell walls, also possess some lysozyme activity hydrolysing the bacterial peptidoglycans (Boller 1988, Minic et al. 1998), indicating that these enzymes may also function in defense against bacteria. Some chitinases are also antifreeze proteins (Yeh et al. 2000). In addition, non-defense roles of chitinases in nodule formation (Goormachtig et al. 1998) and in host specificity of rhizobia (Minic et al. 1998) have been reported in Fabaceae. The isolation of a cDNA clone for the 32 kDa VSP from alfalfa taproots and the alignment analysis of the deduced amino acid sequence reveal that 32 kDa VSP gene is most similar (75% identity) to a class III chitinase isolated from S. rostrata (accession no. Z48671, Goormachtig et al. 1998). Research conducted on chitinase A1 from Bacillus circulans WL-12 showed that the residues Glu-204 and Asp-200 are directly involved in the catalytic mechanism of the enzyme (Watanabe et al. 1993). Although there is only limited sequence homology between the B. circulans chitinase and the alfalfa HMW VSP (12% identity), the Glu and Asp residues (Glu-145; Asp-141; Fig. 7 boxed) corresponding to Glu-204 and Asp-200 are conserved in the alfalfa 32 kDa VSP and other plant type III chitinases. In the present study, we demonstrate that the 32-kDa protein possesses chitin-binding properties with in vitro chitinolytic activity and is specifically recognized by anti-32 kDa VSP antibodies. All together, these results show that the 32 kDa VSP in alfalfa taproot acts as a chitinase. Moreover, the present study indicates that taproot-specific expression of the gene encoding for the 32 kDa VSP in alfalfa was significantly stimulated following MeJA exposure. These data suggested that MeJA is able to induce the expression of a chitinase in alfalfa taproots, which is in agreement with Zhao and Chye (1999) who reported that MeJA induced the expression of a chitinase in Brassica juncea L. Based on these observations, it could be assumed, as previously suggested by Staswick (1994) and Creelman and Mullet (1997), that VSP synthesis might be an adaptive response to wounding and pathogen attack by way of recycling N from damaged tissues. Therefore, it would be interesting to study the effect of taproot wounding or infestation by pathogens on alfalfa VSP accumulation. Future investigations will also need to confirm and understand the putative dual function of this 32 kDa VSP (N storage and chitinase) in response to abiotic or biotic stresses including pathogen attack. This possible dual role was recently demonstrated for other VSPs. For example, Xu et al. (2000) reported that the 18 kDa VSP of Taraxacum officinale Weber possessed properties of a PR protein. Similarly, Yeh et al. (1997) and Tonón et al. (2001) showed that sporamin (VSP of sweet potato tuber) and patatin (VSP of potato tubers) might act as a trypsin inhibitor and a β-1-3 glucanase, respectively. Very recently, Flores et al. (2002) showed that ocatin, a novel tuber storage protein from Oxalis tuberosa Mol, possessed antibacterial and antifungal activities. A major consensus developed from these studies appears to be that VSPs do not exclusively serve as N storage during specific phases of plant development, but also can act in plant defense reactions as PR protein.
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References

Cyr DR, Bewley JD (1990) Proteins in the roots of the perennial weeds chicory (Cichorium intybus L) and dandelion (Taraxacum officinale Weber) are associated with overwintering. Planta 182: 370–374
Kodu Y (1997) Possible involvement of jasmonates in various morphogenetic events. Physiol Plant 100: 639–646
Stephenson LC, Bunker TW, Wesley ED, Grimes HD (1998) Specific soybean lipoygenases localized to discrete subcellular
compartments and their mRNAs are differentially regulated by source-sink status. Plant Physiol 116: 923–933

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