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Nitrogen storage and remobilization in *Brassica napus* L. during the growth cycle: identification, characterization and immunolocalization of a putative taproot storage glycoprotein

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Abstract

In taproot of oilseed rape (*Brassica napus* L.), a 23 kDa polypeptide has been recently identified as a putative vegetative storage protein (VSP) because of its accumulation during flowering and its specific mobilization to sustain grain filling when N uptake is strongly reduced. The objectives were to characterize this protein more precisely and to study the effect of environmental factors (N availability, daylength, temperature, water deficit, wounding) or endogenous signals (methyl jasmonate, abscisic acid) that might change the N source/sink relationships within the plant, and may therefore trigger its accumulation. The 23 kDa putative VSP has two isoforms, is glycosylated and both isoforms share the same N-terminal sequence which had been used to produce specific polyclonal antibodies. Low levels of an immunoreactive protein of 24 kDa were found in leaves and flowers. In taproot, the 23 kDa putative VSP seems to accumulate only in the vacuoles of peripheral cortical parenchyma cells, around the phloem vessels. Among all treatments tested, the accumulation of this protein could only be induced by abscisic acid and methyl jasmonate. When compared to control plants, application of methyl jasmonate reduced N uptake by 89% after 15 d, induced a strong remobilization of N from senescing leaves and a concomitant accumulation of the 23 kDa putative VSP. These results suggested that, in rape, the 23 kDa protein is used as a storage buffer between N losses from senescing leaves promoted by methyl jasmonate and grain filling.

Key words: Abscisic acid, *Brassica napus* L., immunolocalization, methyl jasmonate, nitrogen mobilization, regulation of VSP expression, senescence, VSP accumulation.

Introduction

Specific vegetative storage proteins (VSP) used for the temporary storage of nitrogen have been identified in herbaceous and woody plants, with the frequency of VSP occurrence increasing as more species are investigated. In perennial species, these polypeptides are accumulated during autumn and winter in storage organs and are extensively depleted during spring. Apart from providing N for shoot growth during spring, VSP have also been shown to supply N to the shoot regrowth of forage species such as *Medicago sativa* (Volenec *et al*., 1996) and *Trifolium repens* (Corre *et al*., 1996) following severe defoliation when N uptake and/or fixation are severely inhibited. In annual species like *Glycine max* (Wittenbach, 1983; Staswick, 1992, 1994), VSP are accumulated during flowering and mobilized during seed filling. The soybean VSPα and VSPβ are glycoproteins with a low molecular mass of 27 and 29 kDa, respectively (Wittenbach, 1983). They are stored in vacuoles of a specialized leaf tissue, the paraveinal mesophyll (Franceschi and Giaquinta, 1983; Franceschi *et al*., 1983). These polypeptides usually make up more than 5% of the leaf soluble protein at flowering and are then degraded and presumably contribute to the pool of nitrogen that is mobilized to the developing seeds. On this basis, Wittenbach has called them vegetative storage protein (Wittenbach, 1983). In soybean, these

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proteins are not unique to leaves but are also detected in stems, petioles, pods, roots, and nodules, and in cotyledons after germination but not in seeds (Wittenbach, 1983). In *Arabidopsis thaliana*, VSPs of 29 and 30 kDa homologous to soybean VSP have been characterized (Berger *et al*., 1995), and found mostly in flowers and buds, but their exact role remains unknown.

Most of the information on the regulation of VSP metabolism has been provided by studies on soybean, poplar and *Arabidopsis thaliana*. The expression of VSP appears to be regulated developmentally, by external stimuli such as water deficit, wounding, daylength, temperature, N availability or endogenous signals (like the plant growth regulator jasmonate) that modify N source/sink relationships within the plant. Numerous studies on soybean and *Arabidopsis thaliana* clearly demonstrate that jasmonate and its derivative methyl ester, methyl jasmonate, whose highest levels are found in flowers and reproductive tissues (Creelman and Mullet, 1995), have a significant role in the regulation of VSP genes. Thus, foliar spraying of methyl jasmonate on soybean (Anderson, 1988; Mason and Mullet, 1990; Staswick, 1990, 1992) and application to the roots of *Arabidopsis thaliana* (Berger *et al*., 1995), even at low concentrations, have been shown to increase VSP transcript level. Further evidence implicating endogenous jasmonate in VSP gene regulation comes from studies using inhibitors of lipoxygenase, the first enzyme in the jasmonate biosynthetic pathways (Staswick, 1994). Recently, jasmonate has also been identified as an influential component of the signal-transduction pathway that mediates systemic defence responses through changes in the production of defence-related metabolites such as nicotine accumulation in wounded or herbivore-damaged tobacco plants (Baldwin, 1988*a*, *b*, 1989; Baldwin *et al*., 1994*a*, *b*, 1997).

In previous work (Rossato *et al*., 2001), a protein of 23 kDa has been identified in the taproot of oilseed rape (*Brassica napus* L.) which, by analogy with soybean, could act as a vegetative storage protein (VSP) because of its accumulation during flowering and its specific mobilization to sustain grain filling when N uptake is strongly reduced. Signals involved in the regulation of the expression of this putative VSP are unknown. Previous data showed that its synthesis is probably linked to N made available by foliar senescence. The aims of the present work were to characterize this protein at the biochemical level and to identify putative ellicitors of its accumulation such as N availability, daylength, temperature, water deficit, CO_2 level, and wounding which are known to modify N source/sink relationships within the plant and to regulate VSP gene expression. Furthermore, as the 23 kDa protein synthesis is linked to N made available from leaf senescence (Rossato *et al*., 2001), the effect of different factors supposed to be involved in foliar senescence such as L-serine, ascorbic acid, methyl jasmonate, and abscisic acid has also been investigated. Effects of methyl jasmonate on growth, N partitioning within the plant and on further induction of the putative VSP synthesis were subsequently studied.

Materials and methods

Plant culture

Brassica napus L. cv. Capitol plants were taken from a field plot located near Cully (Calvados, France) in December when they were at the six leaf stage. The roots were gently rinsed with distilled water before transferring the plants to a hydroponic system (27 seedlings per 15 l plastic tank) in a growth room. The aerated nutrient solution previously described (Rossato *et al*., 2001) was renewed every 2 d. $CaCO_3$ was then given in excess at a final concentration of 2 mM in order to maintain the solution pH at 6.5 ± 0.2. Light was provided by high-pressure sodium lamps (250 μmol m^{-2} s^{-1} of photosynthetically active radiation at the height of the canopy) for 16 h d^{-1}. The thermoperiod was 20 °C (day) and 15 °C (night). The plants were used for experiments when the lateral roots (partly damaged during collection of the plants from field plots) had been growing for 3 weeks.

Effect of trophic, environmental and physiological factors on the accumulation of the putative VSP of 23 kDa

At day 0, different treatments which are known to modify N source/sink relationships within the plant and to regulate VSP gene expression, and/or to be involved in foliar senescence, were applied for 10 d (experiment 1) or 15 d (experiment 2). Thus, for experiment 1, plants were submitted to the following six treatments for 10 d: N starvation (no N supplied), salt stress (NaCl, 0.4 M), root application of methyl jasmonate (MeJa, 100 μM, Sigma, Saint-Quentin Fallavier, France), abscisic acid (ABA, 6.2 μM, Sigma, Saint-Quentin Fallavier, France), L-serine (30 mM) or ascorbic acid (100 μM). For experiment 2, the effect of high nitrogen supply (1–50 mM KNO_3), short-day photoperiod (8 h light, 16 h darkness), low leaf temperature (4 °C), reduced CO_2 level (175 ppm), osmotic stress (PEG 6000, 12.87% w/v giving a Ψ_0 in the solution of −0.335 MPa) and leaf wounding (10 pinholes and 10 razor blade lacerations per leaf) were studied for 15 d. For both experiments (1 and 2), the composition of the nutrient solution was identical to the above-described solution except for high N supply (5, 10 and 50 mM KNO_3), and for N starvation where the N source (KNO_3) was removed. Control plants (grown constantly under the same above-described conditions) and treated plants were harvested on the first day of treatment and 10 d or 15 d later, respectively, for experiments 1 and 2.

Effect of methyl jasmonate on N partitioning in plants

At day 0, 100 μM methyl jasmonate was applied in the nutrient solution of the plants for 15 d. Throughout this period, the composition of nutrient solution was identical to the above-described solution except for the N source ($K^{15}NO_3$) which was labelled with a ^{15}N excess of 1.0%. Control plants (grown without methyl jasmonate) and treated plants were sampled after 0 and 15 d of treatment.

Effect of methyl jasmonate on the accumulation of the putative VSP of 23 kDa

At day 0, plants were treated with methyl jasmonate for 10 d. Methyl jasmonate was added to the nutrient solution to a final concentration of 10, 50 or 100 μM, or sprayed daily on the leaves (100 μM MeJa, 0.05% (v/v) Tween 20; 10 ml per plant). 100 μM methyl jasmonate was also added to the nutrient solution in the presence of salicylic acid (1 mM). Control plants were grown under the same above-described conditions without methyl jasmonate treatment. Plants were harvested on the first day of treatment and 10 d later.

Sampling, chemical analysis and calculation of N remobilization

Roots of plants supplied with $K^{15}NO_3$ were first rinsed with a 1 mM solution of $CaSO_4$ to remove any superficial ^{15}N. At each harvest date, 12 plants were separated into leaves, stems, taproots, and lateral roots. Each plant fraction was weighed, freeze-dried, re-weighed for dry weight determination, and then ground to a fine powder for isotopic analysis. For SDS-PAGE and Western blot analysis of the soluble proteins, taproots of harvested plants were immediately frozen in liquid N_2 and kept at −80 °C until soluble protein extraction.

The total N and ^{15}N in the plant samples was determined with a continuous flow isotope mass spectrometer (Twenty-twenty, PDZ Europa Scientific Ltd, Crewe, UK) linked to a C/N analyser (Roboprep CN, PDZ Europa Scientific Ltd, Crewe, UK). As all the mineral N taken up from the nutrient solutions was ^{15}N-labelled, the cumulative uptake and further translocation into plant parts could be calculated from the excess ^{15}N in each tissue. Consequently, the patterns of net translocation of unlabelled N (^{14}N absorbed prior to the beginning of the experiment) between plant parts could be used to calculate N remobilization within the plant. The N in growing leaves derived from the mobilization of endogenous unlabelled N was calculated by subtracting from total N ($^{14}N + ^{15}N$), firstly, the ^{15}N content derived from uptake of $^{15}NO_3^-$ and, secondly, the initial ^{14}N content found in this tissue at the beginning of the experiment.

Soluble protein extraction and analysis

Soluble proteins were extracted and analysed by SDS-PAGE as previously described (Rossato *et al*., 2001). Electrophoretic transfer of proteins from SDS-PAGE gels onto PVDF membrane (Immobilon-P, Proteigene, Saint-Marcel, France) was conducted by semi-dry electroblotting (100 V, 2.5 mA for 30 min, Milli Blot system, Proteigene), according to the protocol described previously (Towbin *et al*., 1979). After blotting, PVDF membranes were treated with affinity-purified polyclonal anti-23 kDa protein (dilution 1/1000) primary antibodies. The antigen–antibody complex was visualized with alkaline phosphatase linked to goat (*Ovis* L.) anti-rabbit (*Oryctolagus cuniculus* L.) IgG as described earlier (Blake *et al*., 1984).

Glycoprotein extraction and analysis

Taproots were harvested from *Brassica napus* L. cv. Capitol plants taken from a field plot located near Cully (Calvados, France) in May when they were at the end of the G1 flowering stage (CETIOM source). Fresh tissue was ground in 80 mM citrate–phosphate buffer, pH 5.5 (1 ml g^{-1} FW), 1 mM DTT, and 0.1 mM PMSF. After filtration through two layers of Miracloth, the extract was centrifuged at 20 000 *g* for 10 min. 1% (w/v) polyvinylpolypyrolidone and 100 mg l^{-1} protamine sulphate were then added to the supernatant. After 10 min, the extract was centrifuged at 20 000 *g* for 10 min. The remaining supernatant was precipitated with 80% (w/v) ammonium sulphate for 20 min, then centrifuged at 27 000 *g* for 20 min. The pellet was dissolved in 80 mM citrate–phosphate buffer (pH 5.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 500 mM NaCl. This solution was applied to a Concanavalin A-Sepharose column (2 × 30 cm) equilibrated with the resuspension buffer. The column was washed with buffer until no more protein was eluted. Then, 500 mM α-D-methylmannopyranoside in the same buffer was added to elute the bound protein. All steps after tissue-collection were performed at 4 °C. Both fractions containing either the bound or the unbound protein were collected, ultracentrifuged (3 × 90 min) through Amicon CP-10 units, then precipitated with acetone for 30 min at −20 °C. After centrifugation (10 min, 18 000 *g*), pellets were dried and stored at −80 °C.

Methods have already been described for two-dimension gel electrophoresis (Rossato *et al*., 2001).

N-terminal sequencing of the putative VSP of 23 kDa, antibody production and purification

The putative VSP of 23 kDa was purified from preparative 2-D SDS-PAGE and then electrophoretically transferred onto a PVDF membrane as described above. The two isoforms of the 23 kDa protein were localized on the membrane after staining the blot with Coomassie Brillant Blue R-250. The two bands of interest were cut from the blot using a razor blade and were analysed for N-terminal microsequencing on 15 amino acids (Institut für Biochemie der Medizinischen Fakultät der Universität Wien). A synthetic peptide was produced using this N-terminal sequence (Eurogentec SA, Seraing, Belgique), and then coupled with a carrier protein 'Keyhole Limpet Hemocyanin', and injected into rabbits. After 4 months of monthly injections, polyclonal antibodies anti-23 kDa were collected and affinity-purified.

Immunostaining for light microscopy

After 10 d of root methyl jasmonate application (100 μM), taproot tissue was excised 2 cm below the stem attachment. Taproots were immunostained for light microscopy as described previously (Avice *et al*., 1996), except that the antibody solution (anti-23 kDa protein IgG) was used at 1/60 dilution. Negative controls were performed to confirm the specific labelling of the sections by substitution of primary antibody with pre-immune serum.

Immunostaining for electron microscopy

Putative 23 kDa VSP immunolocalization was obtained with tissue fixation in 2% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 °C for 2 h, and a tissue polymerization in Unicryl (72 h, 4 °C, illumination with UV light, 360 nm). Ultrathin sections (80 nm) were cut from the taproot prepared as for light microscopy, with an ultramicrotome using a diamond knife and collected on Formvar (0.25% in dichloroethane)-coated nickel grids and immunostained. Immunogold labelling was carried out by first incubating sections at room temperature in 20 μl of 0.1% bovine serum albumin/10% normal goat serum (v/v) in TBS for

20 min. Excess solution was then drained from the sections and replaced with 20 µl of antibody solution (anti-23 kDa protein IgG at 1/60 dilution) in TBS-bovine serum albumin containing 0.05% (v/v) Tween 20 for 1 h at 37 °C. Sections were then washed thoroughly and repeatedly for 5 min in TBS–0.1% bovine serum albumin (pH 7.8) and the area around the sections carefully dried. Sections were immersed in 20 µl goat anti-rabbit IgG conjugated to colloïdal gold (Biocell, 30 nm particle size, 1/100 working solution in TBS-bovine serum albumin 0.1%) for 60 min at room temperature. The grids were then washed thoroughly with TBS followed by distilled water, dried and finally counterstained with 5% aqueous uranyl acetate and lead citrate. Electron microscopy observations were performed on a Siemens Elmiskop 102.

Statistical analysis

Experiments were performed with 12 replicates (one plant per replicate). Results represented the mean ± SE for $n = 12$. For soluble protein analysis (gels and blots), results were given from the combined extracts of three replicates, each containing four plants.

Results

Effect of trophic, environmental and physiological factors on accumulation of the 23 kDa protein in taproot

Rape plants were submitted to different treatments in order to study the effect on accumulation of the 23 kDa protein in taproot, of factors that are known either to modify N source/sink relationships within the plant and to regulate VSP gene expression, or to be involved in foliar senescence. A high N supply (1–50 mM KNO_3) did not induce accumulation of the 23 kDa protein (Fig. 1A). Similarly, accumulation of the 23 kDa protein was not altered by N starvation (Fig. 1A). Accumulation of the 23 kDa protein was neither stimulated by short-day photoperiod nor low foliar temperature (Fig. 1B). Likewise, stress application such as a low CO_2 level, osmotic stress using NaCl or PEG 6000, or foliar wounding did not enhance the accumulation of the 23 kDa protein (Fig. 1B).

Furthermore, as the 23 kDa protein synthesis is linked to N made available from leaf senescence (Rossato *et al.*, 2001), the effect of different factors supposed to be involved in foliar senescence such as L-serine, ascorbic acid, methyl jasmonate, and abscisic acid has been investigated (Fig. 1C). The accumulation of the 23 kDa protein was not enhanced by L-serine or ascorbic acid; it was, however, strongly stimulated by root application of methyl jasmonate, and abscisic acid (Fig. 1C). Thus, after 10 d, this protein represented 16% and 7% of the total soluble protein for methyl jasmonate and abscisic acid-treated plants, respectively, versus 2.4% for control plants (Fig. 1C). It follows that the synthesis of this protein seems to be induced by an external supply of phytohormones involved in foliar senescence such as abscisic acid or methyl jasmonate.

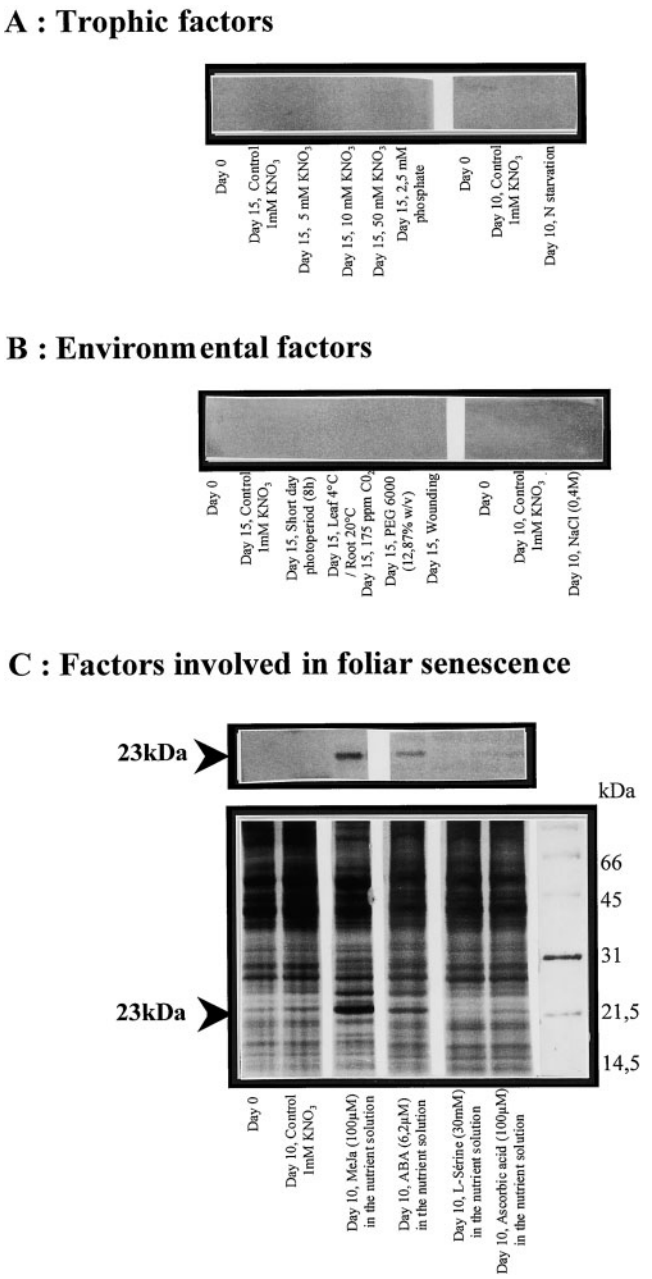

Fig. 1. The effect of trophic factors (A), environmental factors (B) or factors involved in foliar senescence (C) on the accumulation of the 23 kDa putative VSP in *Brassica napus* L. taproot. For SDS-PAGE, each well was loaded with a constant amount of soluble proteins (3.4 µg) and the position of molecular weight markers is indicated on the right. Immunodetection of the 23 kDa protein was performed after separation by SDS-PAGE and Western blotting of the taproot soluble protein. Details about different treatments are given in the Materials and methods. Arrows indicate putative VSP of 23 kDa.

Effect of methyl jasmonate on growth and nitrogen flows

Root application of methyl jasmonate affected the total growth of plants (Fig. 2A). Thus, the total dry matter of plants treated with methyl jasmonate declined by nearly 39% in comparison with control plants (Fig. 2A).

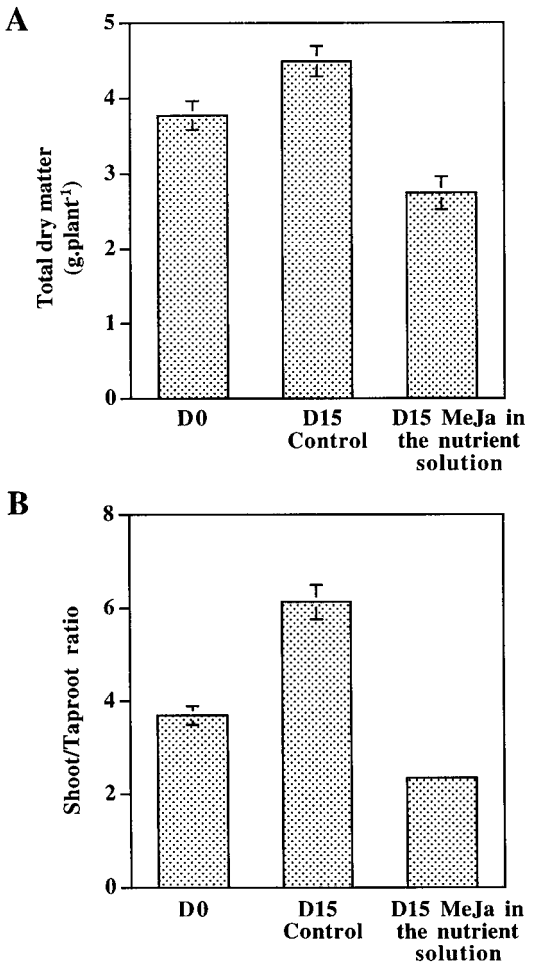

Fig. 2. The effect of methyl jasmonate supply (100 μM) on total dry matter (A) and shoot/root ratio (B). D_0: first day of experiment; D15: 15 d with methyl jasmonate supply (MeJa, 100 μM). Vertical bars, when large enough, indicate ±SE of the mean for $n = 12$.

Furthermore, the shoot–taproot ratio was strongly decreased from 6.1 to 2.3 (Fig. 2B). Methyl jasmonate also reduced $^{15}NO_3^-$ uptake by 89% after 15 d as compared with control plants (Fig. 3). However, plants treated with methyl jasmonate showed a preferential partitioning of ^{15}N taken up to taproot (20.2% versus 5.7%) and lateral roots (12.1% versus 6.3%), at the expense of aerial tissues (Fig. 3). Thus, only 25.4% of absorbed ^{15}N was translocated to the leaves of the treated plants versus 42.7% for control plants. Translocation of ^{15}N taken up to stems was little affected by methyl jasmonate supply (42.3% versus 45.3% for control plants, Fig. 3). The inhibition of $^{15}NO_3^-$ uptake by methyl jasmonate was concomitant with a strong remobilization of endogenous unlabelled N from leaves (24.1 mg ^{14}N corresponding to 47.6% of their initial ^{14}N content) to stems (54.2%), taproots (36.8%) and, to a lesser extent, to lateral roots (9.0%) (Fig. 2). On the other hand, such intense remobilization of endogenous ^{14}N was not found for control plants (Fig. 3).

Effect of methyl jasmonate on the accumulation of the 23 kDa protein

Previous results showed that methyl jasmonate applied in the nutrient solution of the plants stimulated the accumulation of the 23 kDa protein. In order to understand the inducing role of methyl jasmonate on the 23 kDa protein accumulation, plants were treated with methyl jasmonate applied in the nutrient solution at increasing concentrations (10–100 μM) or sprayed on the leaves. Methyl jasmonate was also applied in the nutrient solution in the presence of salicylic acid, an inhibitor of the biosynthesis of methyl jasmonate and its perception pathway.

Increasing the methyl jasmonate concentration from 10 to 100 μM in the nutrient solution resulted in further stimulation of the 23 kDa protein accumulation (Fig. 4). Thus, this protein represented 10%, 13.5% and 16% of the total soluble protein for methyl jasmonate concentrations of 10, 50 and 100 μM, respectively (Fig. 4). Furthermore, the 23 kDa protein was accumulated at the same rate (16% of the total soluble protein) when methyl jasmonate (100 μM) was applied in the nutrient solution or sprayed on the leaves (Fig. 4). Accumulation of the 23 kDa protein by root application of methyl jasmonate was prevented (from 16% to only 3% of the total soluble protein) by the addition of salicylic acid, indicating that the growth regulator MeJa could be a potential inducer of the 23 kDa protein accumulation (Fig. 4).

Biochemical characterization and immunolocalization of the 23 kDa protein

Glycoproteins were extracted from *Brassica napus* L. taproot as described above to determine whether the two 23 kDa isoforms were glycosylated. Both bound (Fig. 5A) and unbound (Fig. 5B) fractions were analysed by 2D SDS-PAGE. Gels patterns showed that the two 23 kDa isoforms were contained in the bound fraction (Fig. 5A). Thus, these two isoforms are glycosylated.

The N-terminal sequence of the two isoforms is given on 15 amino acids in Fig. 6. Except for the amino acid in position 15, both isoforms of pI 5.5 and 5.4 have the same N-terminal sequence, which suggests that it could be the same protein. A search in several databases (Swissprot, Swnew, Swall, Genbank CDS translations, PDB, PIR) revealed a partial homology (90% of similarity on 11 amino acids) with a protein isolated from phosphate-starved roots of *Medicago truncatula* (Harrison *et al.*, unpublished results).

In order to find out whether the 23 kDa protein accumulated in other rape tissues than taproots, Western blots of soluble protein extracts of leaves, stems, flowers, pods, grains, taproots, and lateral roots were incubated with polyclonal antibodies against the 23 kDa protein (Fig. 7). Low levels of an immunoreactive 24 kDa protein

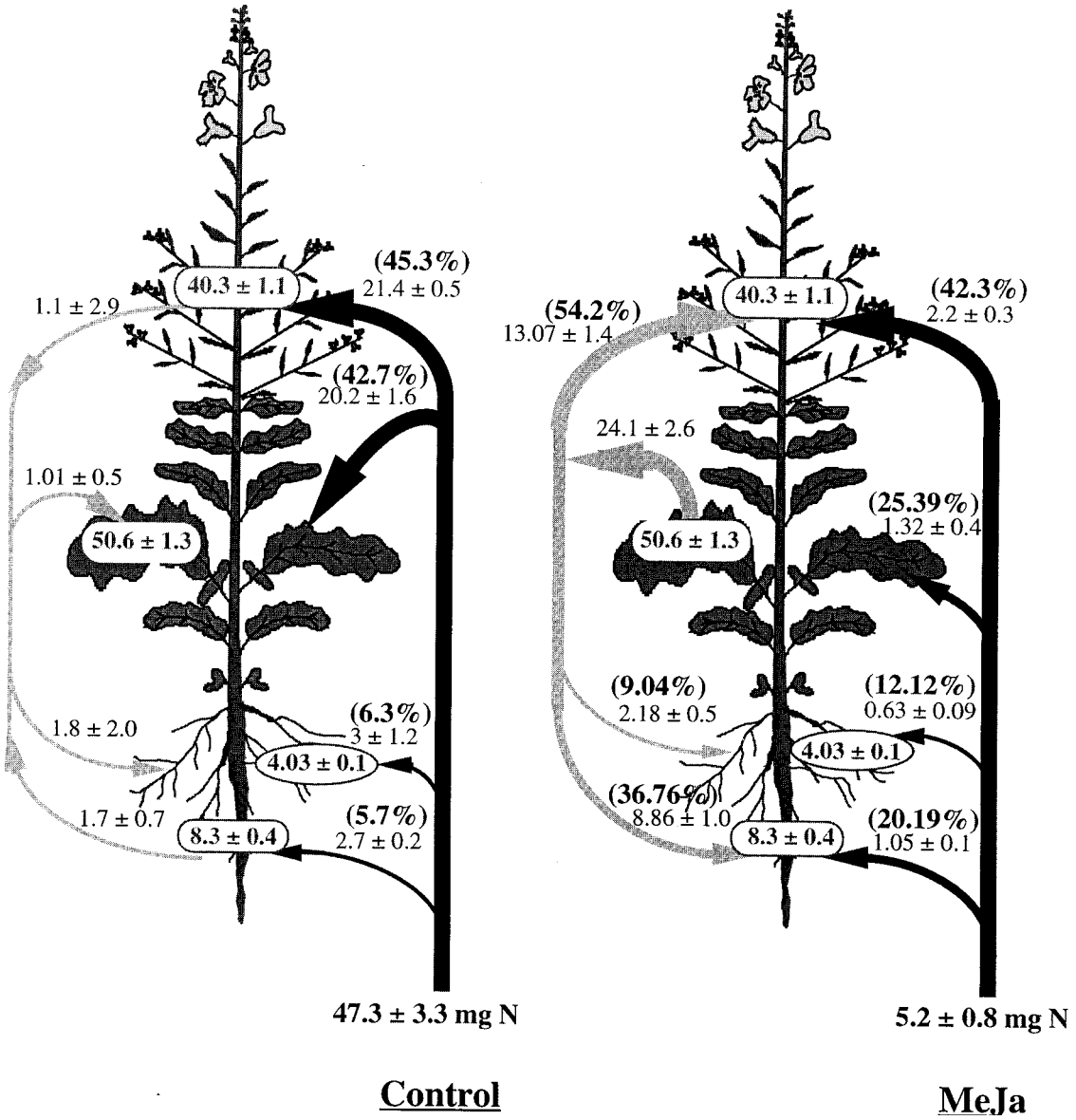

Fig. 3. The effect of methyl jasmonate supply (100 μM) on nitrogen flows within *Brassica napus* L., expressed in mg N plant^{-1} from the internal cycling of unlabelled endogenous N (left arrows) and from the allocation of ^{15}N-nitrate taken up (right arrows), during 15 d of treatment. Each value is given as the mean ± SE of the mean for $n = 12$. Numbers between brackets indicate the percentage of total N taken up, and the total unlabelled endogenous N mobilized from or to each organ. Numbers within ellipses indicate the total N content in each organ at the beginning of the methyl jasmonate treatment.

were found in leaves and flowers whereas no signal was detected in all other tissues tested.

Localization of the 23 kDa protein was achieved using anti-23 kDa polyclonal antibodies and immunogold labelling and microscopy. A pre-immune serum control (Fig. 8A) is shown in which taproot cells are devoid of gold particles, demonstrating the specificity of immunocytochemical labelling. Figure 8B shows that antibodies raised against the 23 kDa protein were only found in peripheral cortical parenchyma cells of *Brassica napus* L. taproot. The 23 kDa protein in peripheral cortical parenchyma cells was detected in vacuoles near starch granules and close to the phloem transport system (Fig. 9). Figure 10 provides additional information regarding intracellular localization of the putative VSP of 23 kDa in peripheral cortical parenchyma cells of *Brassica napus* L. taproot. A gold particle-specific label appeared within the vacuole. The cytoplasm is very slightly labelled as compared with the vacuole whereas the cell wall is unlabelled (Fig. 10). The anti-23 kDa protein antibodies react more specifically with the electron-dense, fibrillar material in the vacuolar compartment (Fig. 10).

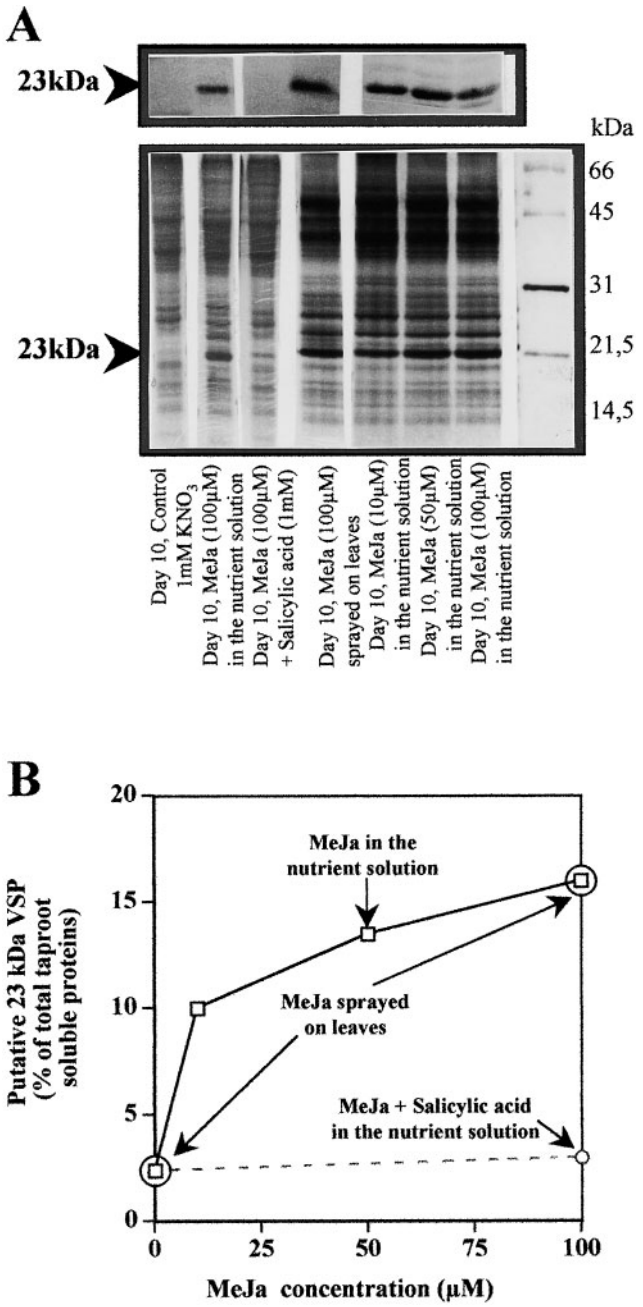

Fig. 4. (A) Effect of methyl jasmonate, applied in the nutrient solution (10, 50 or 100 µM), sprayed on leaves (100 µM), or in the presence of salicylic acid (1 mM), on the accumulation of the 23 kDa putative VSP in *Brassica napus* L. For SDS-PAGE, each well was loaded with a constant amount of soluble proteins (3.4 µg) and the position of molecular weight markers is indicated on the right. Immunodetection of the 23 kDa protein was performed after separation by SDS-PAGE and Western blotting of the taproot soluble protein. Details about different treatments are given in the Materials and methods. Arrows indicate putative VSP of 23 kDa. (B) Putative 23 kDa VSP relative representation given in % of total taproot soluble protein.

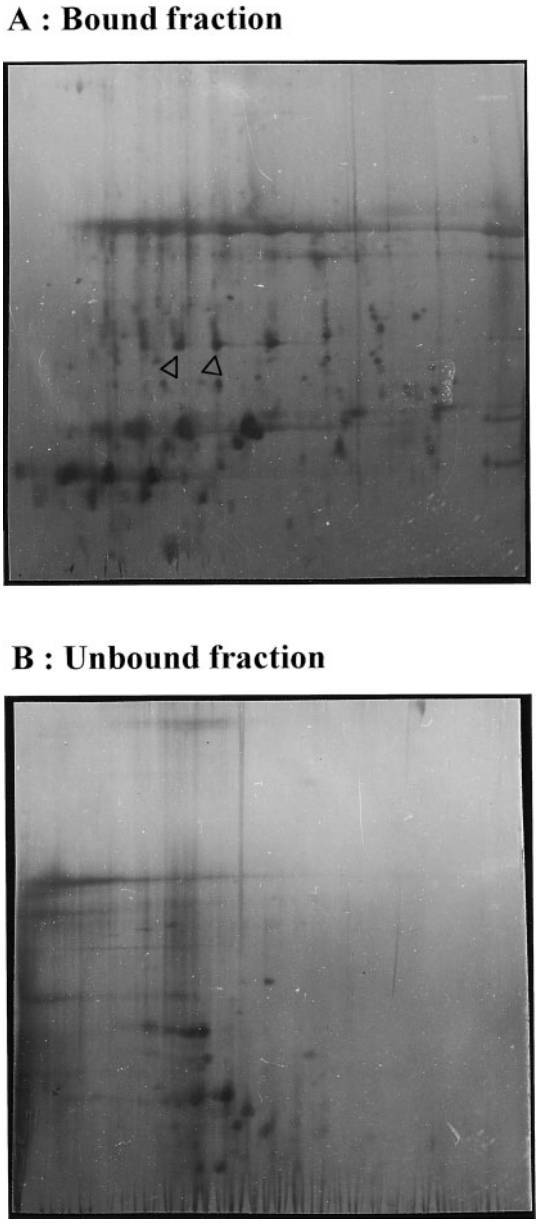

Fig. 5. Two-dimensional gel electrophoresis of soluble proteins extracted from taproots of *Brassica napus* L. at the flowering stage G1 and separated by affinity chromatography on a Concanavalin A-Sepharose column. (A) Bound fraction (glycoproteins). (B) Unbound fraction. Each gel was loaded with 150 µg of soluble proteins and the 23 kDa isoforms are indicated by arrows.

Discussion

These results showed that, like most vegetative storage proteins identified, for example, in woody species (Stépien *et al.*, 1994), soybean (Wittenbach, 1983) or alfalfa (Cunningham and Volenec, 1996), the two 23 kDa isoforms of *Brassica napus* L. taproot are also glycosylated. The role of these oligosaccharide chains remains unclear. It has been proposed that they could confer thermostability during the winter as well as increasing the stored carbon content (Stépien *et al.*, 1994). They are also assimilated to characteristic signals which could facilitate the transit of these VSP through the secretory pathway to the vacuoles (Mason *et al.*, 1988). Moreover, differing

N-terminal sequence of the 23 kDa isoform with pi=5.5

Glu-Glu-Ser-Val-Arg-Asp-Ser-Asn-Gly-Asn-Glu-Val-Val-Leu-Lys
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

N-terminal sequence of the 23 kDa isoform with pi=5.4

Glu-Glu-Ser-Val-Arg-Asp-Ser-Asn-Gly-Asn-Glu-Val-Val-Leu-(Leu, Lys)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 6. N-terminal sequence of the two *Brassica napus* L. 23 kDa putative VSP isoforms of $pI = 5.5$ and 5.4, given on 15 amino acids.

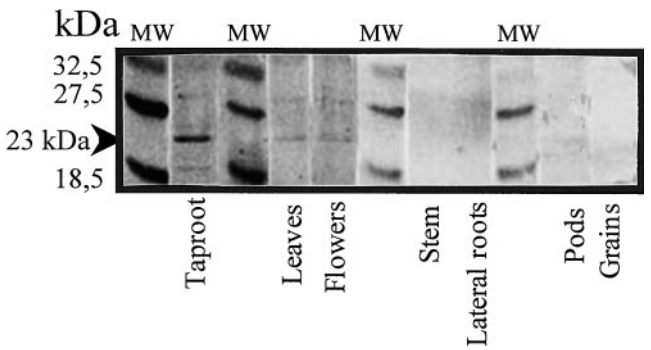

Fig. 7. Immunodetection of the putative 23 kDa VSP in taproot, leaves, flowers, stem, and lateral roots of rape plants at the flowering stage G1, and in pods and grains at the G5 stage (CETIOM source). Values given on the left of the Western blot indicate the molecular weight (MW) of markers. Arrow indicates putative VSP of 23 kDa.

degrees of glycosylation may also explain the occurrence of different isoforms for a given protein, which may be the case for the 23 kDa protein.

The accumulation of VSP in herbaceous and woody species is generally confined to perennial tissues and organs such as roots, trunk, tubers, and stolons (Paiva *et al*., 1983; Hendershot and Volenec, 1993; Stépien *et al*., 1994; Conlan *et al*., 1995; Corre *et al*., 1996), although soybean (Mason *et al*., 1988; Staswick, 1989) and *Arabidopsis thaliana* (Berger *et al*., 1995) provide noticeable exceptions. In rape, Western blot analysis using antibodies raised against the 23 kDa putative VSP showed that this protein was most abundant in taproot and that an immunologically related protein was also detected in leaves and flowers with a molecular weight of 24 kDa.

Furthermore, immunolocalization revealed the presence of the 23 kDa putative VSP only in peripheral cortical parenchyma cells close to the phloem transport system. In soybean, specific accumulation in the paraveinal mesophyll cells has been reported, and it has been argued that deposition of VSP and other N storage compounds adjacent to xylem and phloem facilitates their subsequent mobilization (Staswick, 1994). At the subcellular level, the 23 kDa putative VSP was found more specifically in vacuoles as an electron-dense fibrillar material. This observation is similar to results obtained previously with VSP in poplar wood where the accumulation of fine fibrillar protein material in small vacuoles was demonstrated (Sauter and Van Cleve, 1990).

Among all the treatments tested, accumulation of the 23 kDa putative VSP in the taproot was only stimulated by an external supply of methyl jasmonate or abscisic acid. Methyl jasmonate has been reported to be involved in regulating VSP gene expression endogenously (Staswick, 1990). It is widely distributed in plant species including soybean (Meyer *et al*., 1984). Its effects often mimic those of abscisic acid. Thus, methyl jasmonate, highly active in the range of 1–100 μM, is known to exert various morphological and physiological effects (Sembdner and Parthier, 1993). For example, methyl jasmonate inhibits seed germination, growth of roots and seedlings and flower bud formation (Sembdner and Parthier, 1993). On the contrary, it stimulates fruit ripening, leaf senescence and tuber formation (Pelacho and Mingo-Castel, 1991). Among jasmonate-induced storage proteins, the group of vegetative storage proteins of soybean has been studied intensively in several laboratories. These glycoproteins accumulate in leaves during flowering, leaf senescence and when plants are dessicated (Sembdner and Parthier, 1993). In *Brassica napus* L., the two seed storage proteins (napin and cruciferin) are both induced by methyl jasmonate and abscisic acid (Wilen *et al*., 1991).

If the accumulation of the 23 kDa protein was induced by methyl jasmonate and abscisic acid, which have been found to be signals involved in the wounding and water deficit response processes, respectively, foliar wounding and drought stress have no effect on the synthesis of this protein. In soybean, both methyl jasmonate and wounding have been shown to induce VSP accumulation. Expression of these VSPs is also activated by water deficit, but, surprisingly, not by abscisic acid (Anderson *et al*., 1989), whereas the endogenous level of this phytohormone increases rapidly upon water stress (Zeevaart and Creelman, 1988).

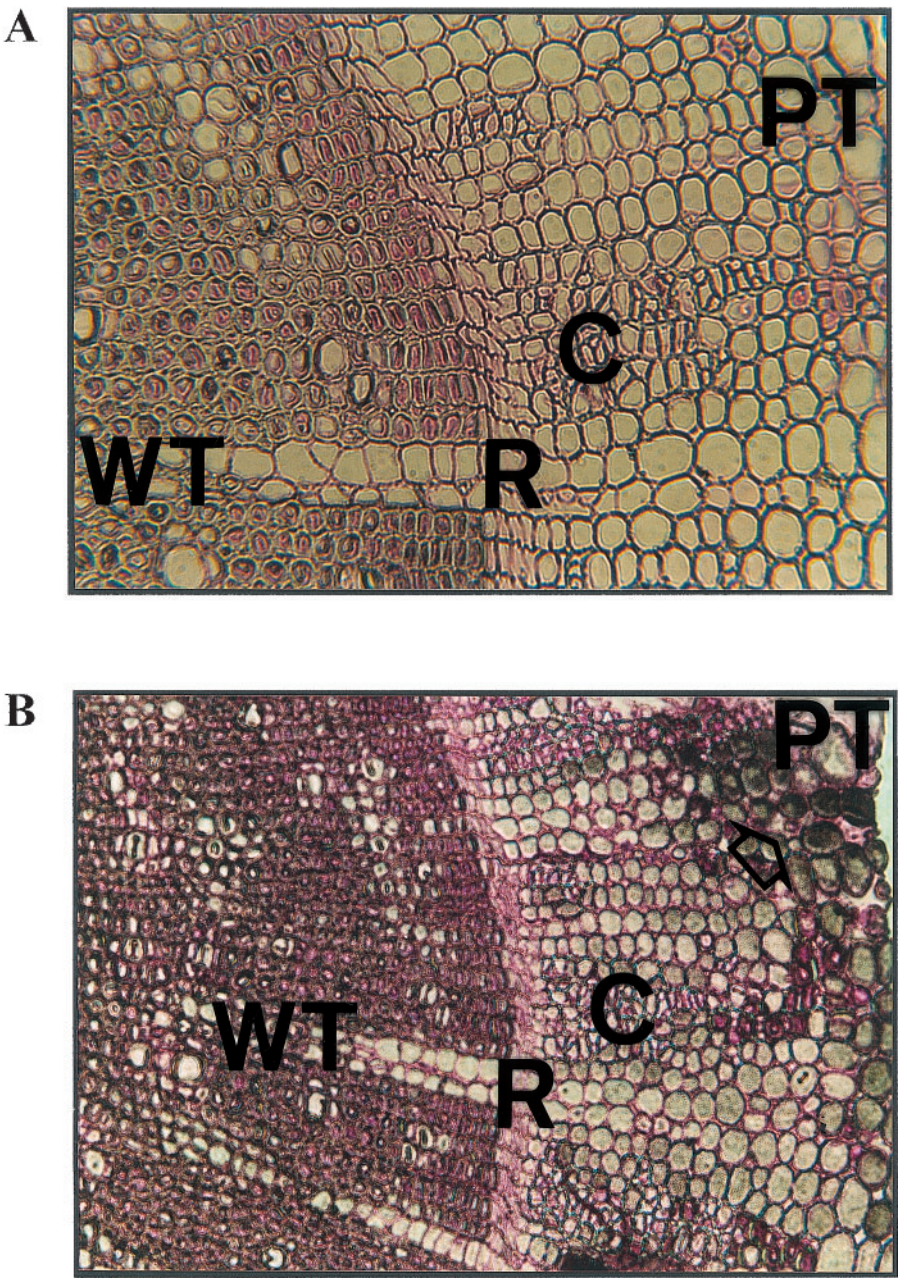

Fig. 8. Tissue immunolocalization of the 23 kDa putative VSP in *Brassica napus* L. taproot treated with methyl jasmonate (100 μM). Transverse taproot sections were treated with pre-immune serum (A) or with antibodies raised against the 23 kDa protein (B). The brown coloration reveals the presence of the 23 kDa protein. PT, phloem tissue; WT, wood tissue; C, cambium; R, medullary ray.

In rape, the increase of N concentration from 1 to 50 mM KNO_3 in nutrient solution did not increase 23 kDa protein accumulation. This contradicts results obtained in soybean (Staswick *et al*., 1991) or poplar (Van Cleve and Apel, 1993) where VSP accumulation significantly increased with increasing N availability. However, because there is a decreased need to mobilize N from mature leaves when N is plentiful, Staswick suggested that the effect of N availability on VSP accumulation in soybean leaves seems related to altered source/sink relations, rather than having a direct regulatory role (Staswick, 1994). On the other hand, it has been reported that VSP accumulation in *Cichorium intybus* L. and *Medicago sativa* L. was unaffected by the increase of N supply (Améziane *et al*., 1997; Noquet *et al*., 2001). This suggests that, like chicory and alfalfa, induction of VSP expression in rape is not directly regulated by inorganic N availability.

Moreover, the results of this study showed that while $^{15}NO_3$ uptake was decreased by 89% after 15 d as compared with a control plant without methyl jasmonate, significant amounts of unlabelled endogenous N were

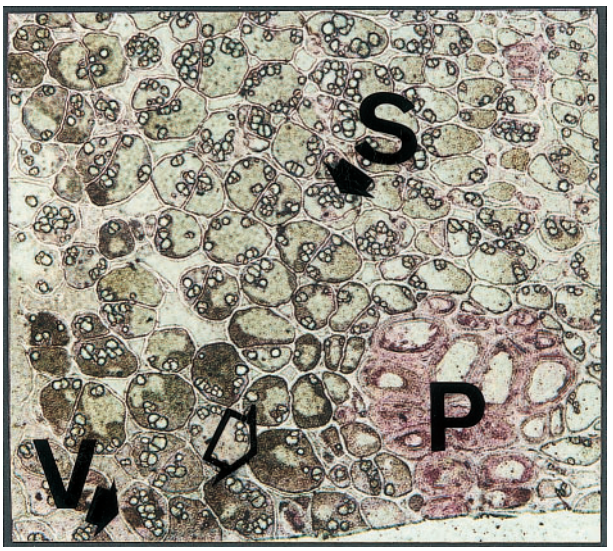

Fig. 9. Tissue immunolocalization of the 23 kDa putative VSP in peripheral cortical parenchyma cells of *Brassica napus* L. taproot treated with methyl jasmonate (100 μM). Transverse taproot sections were treated with antibodies raised against the 23 kDa protein. The brown coloration reveals the presence of the 23 kDa protein. S, starch; P, phloem; V, vacuole.

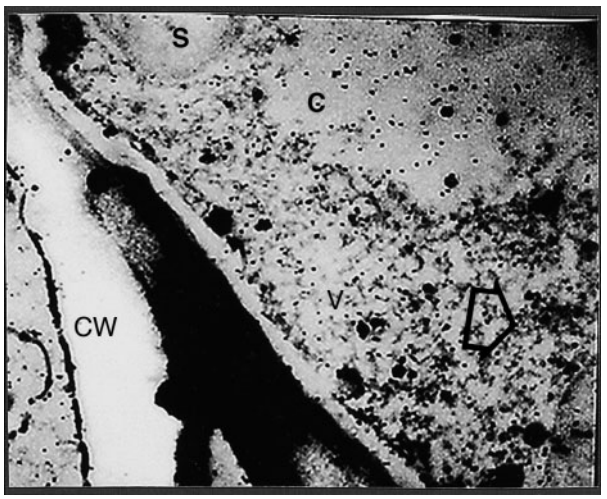

Fig. 10. Intracellular localization of the 23 kDa putative VSP in peripheral cortical parenchyma cells of *Brassica napus* L. taproot treated with methyl jasmonate (100 μM). CW, cell wall; V, vacuole; C, cytoplasm; S, starch.

remobilized from leaves (24.1 mg ^{14}N translocated from green leaves versus 1.3 mg ^{15}N absorbed). Accumulation of the putative VSP of 23 kDa is not stimulated by increased N uptake and, therefore, results more probably from N made available by foliar senescence promoted by hormonal factors only. Thus, other senescence inducers such as L-serine or ascorbic acid, N starvation, PEG 6000, and NaCl had no effect on the induction of the synthesis of the 23 kDa protein although they promoted both growth reduction and mobilization of nitrogen from senescing leaves to taproots, but to a lower extent compared to the methyl jasmonate treatment (data not shown). Such results match those already reported in rape at the flowering stage (Rossato *et al*., 2001) and in chicory where it has also been proposed that the flux of N from leaves to roots may regulate root VSP synthesis (Améziane *et al*., 1997). In rape, methyl jasmonate, a volatile constituent of the floral fragrance, could be considered as a natural signal which promotes foliar senescence and 23 kDa protein accumulation. This hypothesis could be true as it has already been found that the soybean VSPs are induced by atmospheric methyl jasmonate at concentrations estimated to be 40–80 nM or less (Falkenstein *et al*., 1991; Staswick, 1992). Furthermore, the monoterpene *cis*-3-hexen-1-ol, a derivated compound of the jasmonic acid biosynthetic pathway (Creelman and Mullet, 1997) was reported to be one of the major volatile chemicals emitted from oilseed rape in the field at the flowering stage (McEwan and Macfarlane Smith, 1998).

It is concluded that, in rape, the 23 kDa protein is used as a storage buffer between N losses from senescing leaves promoted by methyl jasmonate, and grain filling which appears later. The previous data and literature as a whole taken together appear to be sufficient evidence to ascribe a signal role to methyl jasmonate in the synthesis of the 23 kDa VSP in *Brassica napus* L. Subsequent investigations have, therefore, been performed to study more precisely (on a kinetic basis or along the growth cycle) the effect of methyl jasmonate on N uptake, senescence processes and on the induction of the 23 kDa VSP synthesis in *Brassica napus* L.

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