

The 32-Kilodalton Vegetative Storage Protein of *Salix microstachya* Turz.¹

Characterization and Immunolocalization

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ABSTRACT

A 32-kilodalton vegetative storage protein, found in *Salix microstachya* Turz. bark during the overwintering period, was purified and characterized using several polyacrylamide gel electrophoretic procedures. Solubility characteristics and amino acid analyses were also performed. The protein is water soluble, is glycosylated, has no disulfide-bonded subunits, but is composed of a family of isoelectric isomers. The majority of these isomers are basic. Characteristic of storage proteins, the protein is rich in glutamine/glutamate and asparagine/aspartate (28%), the basic nature of the isomers indicating that most of these amino acid residues are in the amide form. The protein was purified using preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and antibodies raised in chickens. Immunoblot analysis suggested an annual cyclic nature of the accumulation and mobilization of this vegetative storage protein. Immunologically, it is related to a similar molecular weight protein found in the bark of *Populus deltoides* Marsh. but not to any overwintering storage proteins of the other hardwoods tested. Indirect immunolocalization revealed that the protein was sequestered in protein-storage vacuoles in parenchymatous cells of the inner bark tissues of *Salix* during the winter months.

Broadly, storage proteins can be defined as any proteins produced in amounts sufficient to constitute a useful reserve. The storage proteins are degraded and mobilized at a later time, the catabolites supporting the anabolic metabolism associated with new growth (13). Most often this definition is applied to specific proteins found in abundance in seeds. However, it is becoming increasingly evident that particular proteins also play a major role in vegetative nitrogen storage and cycling phenomena in both herbaceous annuals and woody perennials. Specific proteins are synthesized and sequestered within particular vegetative cells in response to certain environmental, physiological, or developmental cues. These proteins are subsequently catabolized and mobilized from the vegetative tissues, the products supporting the ana-

bolic metabolism associated with new, rapid growth. These proteins, therefore, have been classified as vegetative storage proteins (19).

Vegetative storage proteins have been isolated from various plant organs including soybean pods and leaves (19), potato and yam tubers (7, 16), and roots of perennial weeds (4) and from the wood, bark, and leaves of temperate trees (6, 8, 17, 21, 24, 26). In general, they are not homologous to seed storage proteins of the same species (19). Vegetative storage proteins have been localized in protein-storage vacuoles within parenchymal cells of vegetative tissues (6, 8, 23), the organelles being similar in both ontogeny and structure to protein bodies found in mature dicotyledonous seed storage parenchymal cells (5).

In herbaceous annuals, vegetative storage protein accumulation is not restricted temporally. Rather, it is controlled by the nitrogen requirement of other portions of the plant (20). However, in perennial woody species the proteins appear to cycle annually. They are degraded and mobilized in the spring in support of early growth and accumulate in the fall either through increased synthesis or decreased degradation or mobilization. Synthesis during the fall is presumably being supported by the catabolism of leaf proteins and mobilization of amino acids to the retained shoot tissues. In *Salix*, a protein of M_r 32,000 acts as a vegetative storage protein accounting for approximately 30% of the total protein content of the bark tissue during the overwintering period and <5% of the total protein during summer (24). Accumulation of this storage protein coincides with the appearance of protein-storage vacuoles in the cambial cells, phloem parenchyma, and cortical parenchymal cells of the inner bark during the late summer and fall (5, 25). We now confirm its localization in the protein-storage vacuoles and report some of the characteristics of the *Salix* vegetative storage protein.

MATERIALS AND METHODS

Plant Material

The primary material for this investigation was obtained from moderately fertilized mature trees of *Salix microstachya* Turz., grown in the University of Guelph arboretum. Samples of 2- to 4-year-old shoots were collected on a monthly basis from May 1989 to April 1990. The material was either ana-

¹ Supported by the Natural Sciences and Engineering Research Council of Canada and by the Canada, Ontario Forest Resource Development Agreement.

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lyzed immediately or frozen at -70°C for 1 to 3 weeks before analysis.

SDS-PAGE

Proteins were extracted from the bark tissue as previously described (24) and total amounts measured using the Bradford reagent (Bio-Rad Laboratories). Denaturing SDS-PAGE was performed according to the method of Laemmli (12), following instructions given in the Bio-Rad Mini-Protein II cell manual using 12 or 15% SDS-polyacrylamide gels. Loading was on an equal protein basis of $20\ \mu\text{g}$ per lane. For denaturing gel electrophoresis under reducing conditions, β -mercaptoethanol was added to the sample buffer (0.065 M Tris-HCl, 10% [v/v] glycerol, 2% SDS, pH 6.8) to a final concentration of 5% during sample preparation.

Electrophoretic Methods for Additional Characterization

Proteins were extracted from winter bark samples of *Salix* for these analyses. To determine the presence of any disulfide-bonded subunits, a two-dimensional gel system was used (11). After the sample was separated in the first dimension (non-reducing, SDS without $[-]$ β -mercaptoethanol), as described above, a vertical gel slice was incubated for 30 min in the same sample buffer but with the addition of β -mercaptoethanol to 5% (v/v). The gel piece was then placed lengthwise on the 7% stacking gel (no wells) of the second dimension (reducing, SDS with $[+]$ β -mercaptoethanol) gel. The second dimension was run as described above.

Isoelectric focusing in the first dimension using a full pH range, followed by SDS-PAGE in the second dimension, resulted in a complete loss of the M_r 32,000 peptides of interest. Instead, two-dimensional gel electrophoresis, using NEPHGE³ in the first dimension, was performed to determine the isoelectric characteristics of the *Salix* vegetative storage protein (14). Soluble proteins were initially extracted from the bark tissues using 50 mM sodium borate buffer and 1 mM PMSF, pH 9.0, and then washed and concentrated in an Amicon pressure cell (Amicon Corp., Denver, MA) equipped with a PM-10 membrane, using the same buffer. Urea was added to aliquots containing $100\ \mu\text{g}$ of protein to a concentration of 9 M; then, the sample was mixed with an equal volume of 9.5 M urea, 2% (v/v) Nonidet P-40, 2% (v/v) ampholytes (1.6% pH range 5–7, 0.4% pH range 3–10, Bio-Rad), and 5% β -mercaptoethanol. NEPHGE gels were made according to the procedure of O'Farrell *et al.* (14), using a 3% final concentration of ampholytes (pH 5–7) and a 1% final concentration of ampholytes (pH 3–10). A time series of NEPHGE gels containing identical samples was performed and an optimum running time for the protein sample of 2 h at a constant 400 V was determined. For the second dimension, NEPHGE gels were incubated in SDS-PAGE sample buffer with 5% β -mercaptoethanol for a minimum of 20 min, then layered on a 15% SDS-polyacrylamide gel, and run in the second dimension.

To investigate whether proteins were glycosylated, the periodic acid-Schiff staining procedure was used (27).

³ Abbreviation: NEPHGE, nonequilibrium pH gradient electrophoresis.

Solubility Characteristics

To determine solubility characteristics of the *Salix* vegetative storage protein, bark samples were extracted sequentially using deionized distilled water, 0.5 M NaCl, 70% 2-propanol, 60% glacial acetic acid, and 0.1 M NaOH and finally in 0.1 M sodium borate, 1% SDS, and 1% β -mercaptoethanol, pH 10 (9). During the first extraction, water-hydrated polyvinylpyrrolidone was added (2:1, w/w) to reduce phenolic interference, and in all subsequent extractions, 1% PMSF was added to the buffers to inhibit protease activity.

Protein Purification and Antibody Production

To purify protein, SDS-PAGE gels of the total protein extract from winter *Salix* bark samples were run on a Bio-Rad Protein II system. Gels were stained using the Coomassie blue mixture for 10 min to make the storage protein visible, and the 32-kD protein band was cut from the gel. Protein was then eluted from the gel pieces with a Bio-Rad gel eluter into a Tris-glycine buffer (0.024 M Tris base and 0.192 M glycine, 0.1% SDS, pH 8.3). Purity was checked by electrophoresis of an aliquot of the electroeluted protein. For immunization, 50 to $100\ \mu\text{g}$ of purified protein was mixed with Freund's incomplete adjuvant to obtain approximately 1 mL of thick emulsion. One-half milliliter of the suspension was injected into each breast muscle of a laying chicken hen once a week for a total of five injections. After the fifth week, eggs were collected and the IgG was extracted and purified from the yolks according to Jensenius *et al.* (10).

Controls consisted of preimmune eggs and were extracted in the same manner as the immune eggs.

Amino Acid Analysis

Following electroelution, the purified *Salix* vegetative storage protein was exhaustively dialyzed against distilled water and lyophilized. Amino acid analysis was performed by ion-exchange HPLC using a Beckman System Gold Amino Acid Analyzer equipped with postcolumn ninhydrin detection. Samples for analysis were treated with 200 μL performic acid for 3 h at room temperature, dried, and hydrolyzed with 5.7 M HCl at 108°C for 24 h *in vacuo*.

Immunoassays

Western blot analysis was performed using Bio-Rad's mini-blot apparatus according to the method of Towbin *et al.* (22). Localization of bound alkaline phosphatase-conjugated antibodies was performed using the Bio-Rad alkaline phosphatase color development kit, according to manufacturer's instructions. Controls were performed identically using IgG purified from preimmune eggs.

To test whether the vegetative storage protein of *Salix* was related immunologically to those found in other genera, winter bark samples of *Acer saccharum* Marsh., *Populus deltoides* Marsh., *Fagus sylvatica* L., *Fraxinus americana* L., *Gleditsia triacanthos* L., *Robinia pseudoacacia* L., *Tilia americana* L., *Alnus glutinosa* L. Gaertn., *Betula papyrifera* Marsh., and *Quercus rubra* L. were extracted for total protein measurement, proteins were separated by SDS-PAGE, and the sepa-



Figure 1. Western immunoblot of monthly protein samples from bark of *Salix microstachya* probed with chicken anti-*S. microstachya* vegetative storage protein IgG. Samples were loaded on an equal protein per g fresh weight basis. Months are labeled starting with May on the left. Although the vegetative storage protein is present in the bark tissues year-round, substantial accumulation begins in October, with the majority of the protein being mobilized between March and April. Note also that the antibodies detect a doublet from November through March.

rated proteins were transferred to nitrocellulose and immunoblotted according to the above procedures.

Light Microscopy Immunolabeling

Summer and winter bark samples of *Salix* were fixed in mixed aldehydes, dehydrated, and embedded in glycol methacrylate according to the procedures described by Wetzel *et al.* (25). Immunolabeling was performed according to the method of Craig and Goodchild (3). Affinity-purified rabbit-anti-chicken immunoglobulin (IgG, Bio-Rad) was conjugated to 12-nm colloidal gold particles used routinely in this laboratory and the solution purified (18). Silver enhancement of bound colloidal gold was accomplished using the Western blotting grade gold enhancement kit supplied by Bio-Rad, following their instructions. Sections were observed using differential interference contrast (Nomarski) and bright-field optics on a Zeiss-Jena Jenalumar contrast microscope (Jena, Germany). Sections treated identically, but using preimmune IgG, acted as controls.

RESULTS

Seasonal Fluctuations in *Salix* Vegetative Storage Protein

Figure 1 illustrates a Western blot of a SDS gel of monthly *Salix* samples loaded on an equal protein basis and probed with the chicken IgG made against electrophoretically purified *Salix* vegetative storage protein. Only proteins of M_r 32,000 are immunodetected, indicating that the IgG is specific to the *Salix* vegetative storage protein. Control immunoblots, incubated with pre-immune IgG gave no cross-reactivity with any proteins from the *Salix* extracts.

Figure 1 also illustrates the seasonal nature of the accumulation and mobilization of the *Salix* vegetative storage protein. It is present from May to August as a single 32-kD species, although amounts are greatly reduced compared with

late fall and winter months. Accumulation is most rapid between October and November, and levels remain high through the remaining winter months until the protein is mobilized in April. Note also that the antibodies are detecting a second protein of M_r 34,000 in the samples from November to March. Whether this protein has any homology with the M_r 32,000 vegetative storage protein or is a precursor has not been determined, but it is expressed in the same manner.

The 32-kD *Salix* Vegetative Storage Protein Is Like Albumin

It is evident from the Western blot analyses of the various fractions from the sequential extraction procedure (Fig. 2) that the 32-kD *Salix* vegetative storage protein is water soluble and, thus, may be classified as like albumin. Although each extraction was exhaustive, the protein was found to be soluble in distilled water (lane a), acetic acid (lane d), NaOH (lane e), and sodium borate (lane f). However, when the latter three extracts were dialyzed against water, the protein remained in solution. Extractions at high and low pH may have reduced phenolic binding, thereby releasing proteins into solution.

Salix Vegetative Storage Protein Is a Glycoprotein

Periodic acid-Schiff staining of SDS-PAGE-separated proteins indicated that the *Salix* vegetative storage protein is glycosylated (Fig. 3). As a standard to test the specificity and intensity of the staining reaction, Bio-Rad SDS low mol wt markers were used. Only ovalbumin, a known glycoprotein of M_r 45,000, stained, whereas the other nonglycosylated mol wt markers did not.

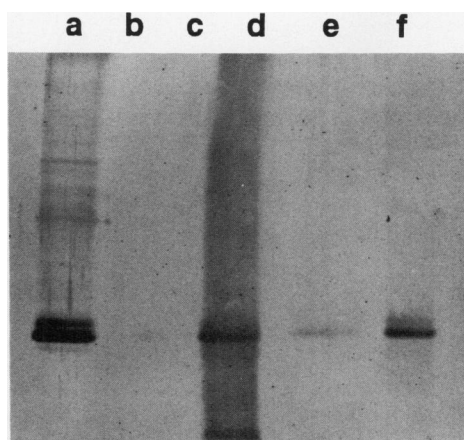


Figure 2. Western immunoblot of sequential protein extractions of overwintering *S. microstachya* bark probed with the vegetative storage protein-specific IgG. Lane a, water-soluble proteins; b, 0.5 M NaCl-soluble proteins; c, 70% 2-propanol-soluble proteins; d, 60% acetic acid-soluble proteins; e, 0.1 M NaOH-soluble proteins; f, 0.1 M sodium borate, 1% SDS, 1% β -mercaptoethanol-soluble proteins. Note that, although the vegetative storage protein is soluble in water, it is also soluble in subsequent solutions.

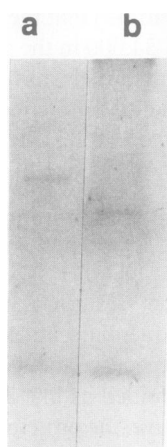


Figure 3. Periodic acid-Schiff-stained SDS polyacrylamide gel of *S. microstachya* overwintering bark extract. Lane a, mol wt markers; b, bark extract. Of the mol wt markers, only ovalbumin (M_r 45,000), a known glycoprotein, gave a positive reaction. The *Salix* vegetative storage protein also gave a positive reaction and is, thus, a glycoprotein.

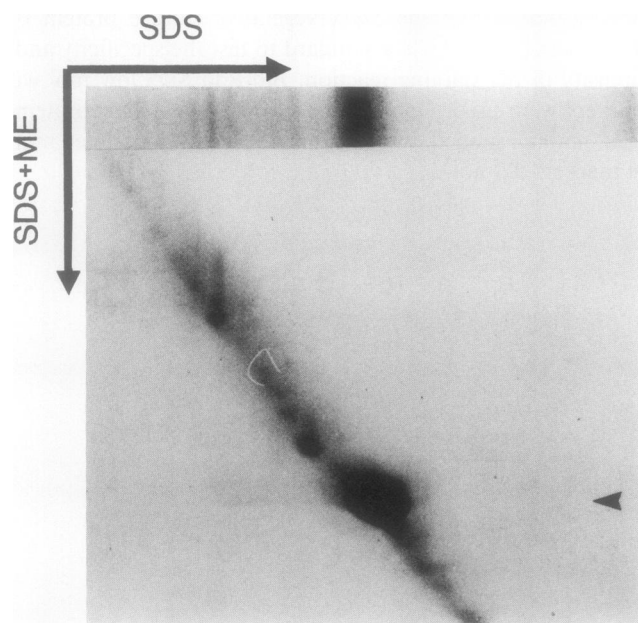


Figure 4. Two-dimensional SDS-PAGE analysis of bark extracts from overwintering *S. microstachya* for determination of presence of disulfide-bonded subunits. Separated proteins were nonreduced in the first dimension and reduced in the second. Disulfide-bonded subunits would move off the diagonal under these conditions. The *Salix* vegetative storage protein (arrowhead) does not contain any disulfide-bonded subunits.

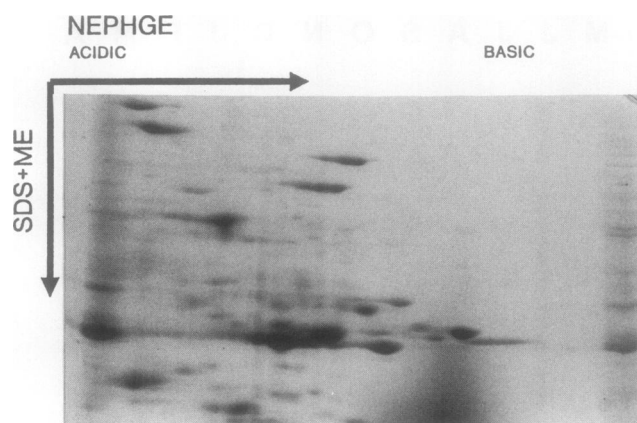


Figure 5. NEPHGE followed by SDS-PAGE of protein extract from overwintering bark of *S. microstachya*. There are seven to 10 isoelectric isomers of the *Salix* vegetative storage protein (arrowheads), the majority of these being basic. Lane a, protein extract of *Salix* bark run in the second dimension for reference.

***Salix* Vegetative Storage Protein Lacks Disulfide-Bonded Subunits**

A two-dimensional SDS-PAGE technique was used in which the first dimension is performed under nonreducing conditions, followed by electrophoresis in the second dimension using reducing agents. Disulfide-bonded protein subunits would be dissociated in the second dimension, resulting in a migration of the resultant subunits to positions below the diagonal (11) (Fig. 4). The *Salix* storage protein does not contain any disulfide-bonded subunits as shown by the absence of any polypeptides below the diagonal.

***Salix* Vegetative Storage Protein Has a Number of Isoelectric Isomers, the Majority Being Basic**

Nonequilibrium PAGE followed by SDS-PAGE separated a number of polypeptides at M_r 32,000 (Fig. 5). Although this group of polypeptides appears to span the entire pH range, the majority of the isoelectric isomers are basic and do not migrate into a regular isoelectric focusing gel (data not shown). For simplicity, we refer to this group as one protein. The amount of homology between these polypeptides is unknown at present.

Amino Acid Analysis of *Salix* Vegetative Storage Protein

Amino acid analysis of electrophoretically purified *Salix* vegetative storage protein demonstrated that it is high in glutamine/glutamic acid and asparagine/aspartic acid (Table I), collectively making up a total of approximately 25% of the amino acid residues. The sulfur-containing amino acids methionine and cysteine constitute approximately 6% of the total.

Immuno-Relatedness of *Salix* Vegetative Storage Protein to Proteins from Other Genera

Immunoblot analysis of proteinaceous extracts from overwintering bark of a number of hardwood species, using the

Table 1. Amino Acid Composition of *Salix microstachya* Vegetative Storage Protein

sd of three trials is given in parentheses.

Amino Acid	% Composition
Ala	8.5 (0.2)
Val	6.7 (0.2)
Leu, nonpolar	9.2 (0.6)
Ile	4.7 (0.1)
Phe	7.6 (2.2)
Met ^a	5.6 (0.9)
Gly	4.4 (4.6)
Ser, polar, uncharged	10.0 (0.0)
Thr	6.3 (0.2)
Cys ^b	1.7 (0.1)
Lys	6.9 (0.6)
Arg, basic	2.7 (0.6)
His	0.5 (0.0)
Asx ^c , basic	12.0 (0.5)
Glx ^d , acidic	13.4 (0.8)

^a Determined as methionine sulfone. ^b Determined as cysteic acid. ^c Aspartate and asparagine. ^d Glutamate and glutamine.

antibodies raised against *Salix* vegetative storage protein, was performed (Fig. 6). The antibodies reacted only with similar M_r proteins found in *P. deltoides*, the only other member of Saliaceae used in this study. There was no cross-reactivity with proteins of other genera.

Immunolocalization of *Salix* Vegetative Storage Protein

The 32-kD storage protein of *Salix* is localized in the protein-storage vacuoles of the phloem parenchyma, cambium, and ray parenchyma of *Salix* shoots during winter (Fig. 7, A and B). Labeling, although present, is relatively sparse in the xylem ray and cambial tissues and is most pronounced in the phloem parenchyma. The vegetative storage protein is restricted to protein-storage vacuoles within each of these cell types. The vegetative storage protein is not as prevalent during summer. Occasionally, protein-storage vacuoles were found in some cells and, when present, gave a light but positive signal for the presence of the vegetative storage protein (Fig. 7, D and E). Labeling is not seen in the control sections from winter (Fig. 7C) or summer bark tissues (Fig. 7F) treated with preimmune IgG.

DISCUSSION

Immunolocalization of the *Salix* vegetative storage protein confirmed earlier indications from ultrastructural studies of seasonal changes in phloem parenchymal cells (5, 25) that protein-storage vacuoles of the overwintering phloem and ray parenchyma were the sites of accumulation. This agrees with past localization studies of vegetative storage proteins in parenchymal cell vacuoles in other genera (6, 8, 19, 23).

The major role of the *Salix* vegetative storage protein appears to be as an overwintering nitrogen reserve. Both SDS-PAGE (24) and immunoblot analysis (this study) suggest an annual cyclic nature of accumulation and mobilization, although it has not been conclusively demonstrated that increased synthesis and accumulation rather than decreased mobilization are occurring at this time. The protein is present

in the inner bark tissues during the summer months, albeit in quantities much reduced compared with those of fall and winter. The immunocytochemical localization studies again confirm this finding: the protein is localized in vacuoles in the phloem parenchymal cells during the summer months, but the frequency of protein-storage vacuoles in any given cell and, indeed, the frequency of cells involved in sequestering the protein during the summer is very low.

The presence of the *Salix* vegetative storage protein in the summer bark could be a function of nitrogen availability. The trees received some exogenous nitrogen during the summer, more so than would be expected in a natural forest stand. Staswick (20) demonstrated that vegetative storage proteins accumulated in other organs if major nitrogen sinks (*i.e.* pods with developing seeds) were removed, *i.e.* if the plant were experimentally placed into a nitrogen surplus situation. Similarly, one could speculate that trees may accumulate vegetative storage proteins during situations of excess nitrogen. Storage protein accumulation may primarily occur when vegetative growth has stopped but could also occur at other times of increased nitrogen availability. This concurs with the findings of Herman *et al.* (8), who localized a vegetative storage protein in vacuoles of leaf mesophyll cells of *Saphora*, a leguminous tree that would not be expected to encounter nitrogen-deficit situations. Sauter *et al.* (17), however, found that the *Populus* vegetative storage protein was completely absent from summer wood samples. This may have been due to limited nitrogen availability or to the extent of the role of the xylem parenchymal cells in vegetative storage protein sequestering.

It is interesting to note the similarity of the *Salix* vegetative storage protein with the *Populus* bark protein; both genera belong to the same family and both contain storage proteins of similar weight (24). This agrees with results of others. Antiserum to soybean vegetative proteins cross-reacts with those of other legumes (19), and patatin, a vegetative storage

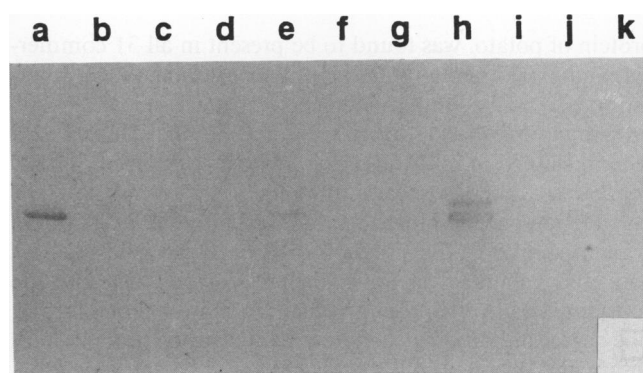
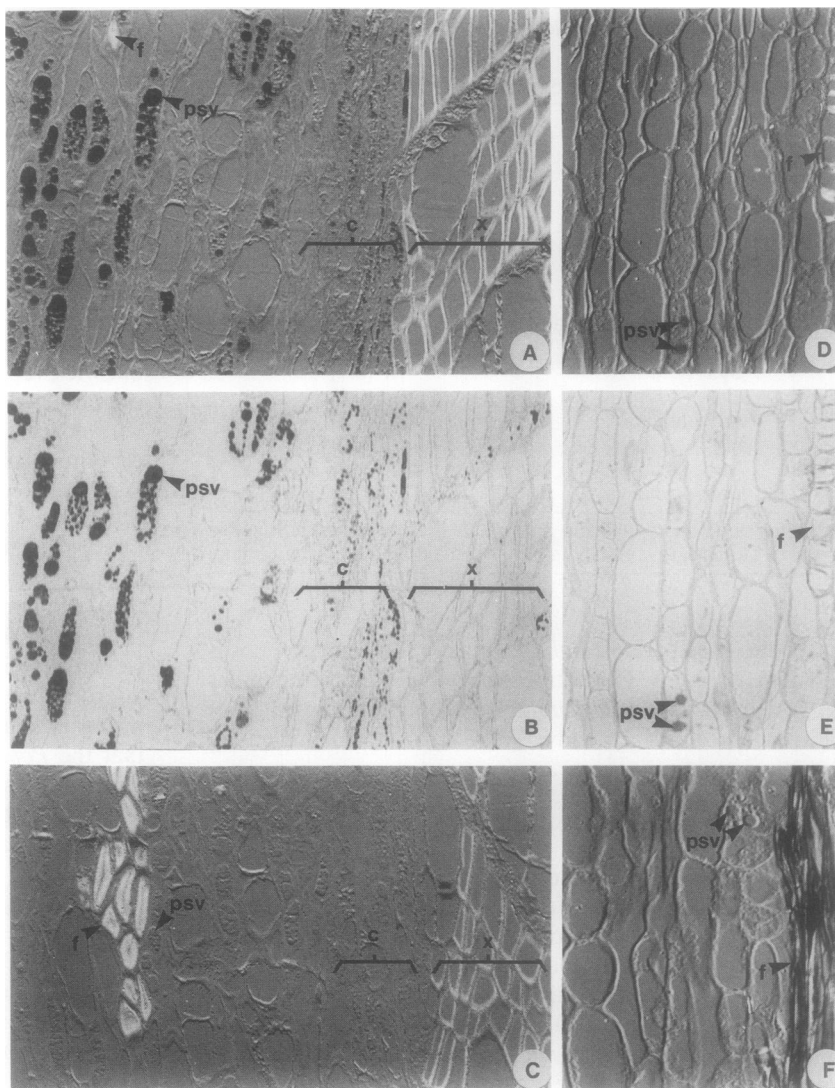


Figure 6. Immuno-relatedness of *Salix* vegetative storage proteins with those of other genera. Immunoblot analysis of protein extracts from overwintering bark of *S. microstachya* (lane a); *A. saccharum* (b); *A. glutinosa* (c); *B. papyrifera* (d); *F. sylvatica* (e); *F. americana* (f); *G. triacanthos* (g); *P. deltoides* (h); *Q. rubra* (i); *R. pseudoacacia* (j); *T. americana* (k) probed with chicken anti-*Salix* vegetative storage protein IgG. Note that a similar M_r protein of *P. deltoides* is the only immunoreactive protein. *Salix* and *Populus* are the only members of Saliaceae used in this study.

Figure 7. Immunolocalization of *Salix* vegetative storage protein. A to C, Radial sections of overwintering *S. microstachya* shoots illustrating the cambial zone (c) and the xylem region (x). Phloem tissue lies to the left of the cambial region. A and B, Section treated with anti-*Salix* vegetative storage protein IgG seen under Nomarski (A) and bright-field (B) optics. Protein-storage vacuoles (psv) in the phloem parenchymal cells are densely labeled, indicating the presence of the vegetative storage protein. Note in B that small protein-storage vacuoles in the cambial cells and xylem ray parenchyma are also labeled. C, As in A and B but treated with preimmune IgG as control and viewed under Nomarski optics. No labeling is seen. D to F, Radial sections of *Salix microstachya* shoots from summer, concentrating on the phloem parenchyma. D and E, Section treated with anti-*Salix* vegetative storage protein IgG seen under Nomarski (D) and bright-field (E) optics. A weak positive signal, indicating the presence of the vegetative storage protein, is seen over occasional protein-storage vacuoles. f, fiber. F, As in D and E but treated with preimmune IgG as control and viewed under Nomarski optics. All micrographs are 400 \times ; bar, 20 μ m.



protein of potato, was found to be present in all 31 commercial and experimental potato cultivars examined (16). Interesting also is the immunodetection of a second M_r 34,000 protein in *Salix* bark during the winter months. This was not seen in stained SDS-PAGE gels of winter samples of *Salix* \times *smithiana*, although species differences may exist (24). The possible presence of more than one vegetative storage protein is again similar to the situation in *Populus* in which at least two, and as many as four, distinct polypeptides may be considered as overwintering vegetative storage proteins (24). The degree of homology between the individual proteins both within and between each species has yet to be determined.

According to the classification of Osborne and Mendel (15) used for seed proteins, the *Salix* vegetative storage protein is an albumin, although the classification may not be appropriate to non-seed proteins. However, additional vegetative storage protein was released in other solvents following exhaustive extraction in water. It is possible that a portion of the vegetative storage protein binds to phenolic compounds or other water-insoluble polymers and are subsequently released when exposed to altered pH conditions. That the protein remains

soluble in water following extensive dialysis of the acidic and basic extractions supports this conclusion. Alternatively, the *Salix* storage protein may be composed of several polypeptides of the same mol wt that have different solubility characteristics (9).

The *Salix* vegetative storage protein is glycosylated, although the nature of the oligosaccharide side chain(s) has not been determined. As with other glycosylated vegetative storage proteins (8, 16, 19), the function of the glycosylation is not understood. The protein is also comprised of seven to 10 isoelectric isomers. The majority of these isomers have a negative charge, moving toward the cathode during NEPHGE. Standard isoelectric focusing procedures failed to resolve any proteins in the 32-kD range. Because the majority of the charge isomers are basic, and, *in toto*, Asx and Glx residues account for approximately 25% of the amino acid residues of the protein, it is highly probable that most of these residues exist in the amide form. Asx and Glx are frequent in seed and vegetative storage proteins, and the amino acid composition of *Salix* vegetative storage protein is similar to those of *Sambucus nigra* (1), soybean (19), yam tuber (7), and

patatin (16). The lack of disulfide subunits is consistent with the relatively low amount of cysteine (1.5%). The presence of the high amounts of the essential amino acids leucine and lysine as well as the sulfur-containing amino acid methionine may explain why *Salix* bark is grazed by animals during the winter. Because of the similarity of *Salix* vegetative storage protein to that of *Populus*, overwintering *Salix* bark may have commercial value as an alternate animal food source (2).

ACKNOWLEDGMENTS

The authors wish to thank Dr. Anthony Clarke, Department of Microbiology, University of Guelph, for performing the amino acid analyses, and Dave Bantroch and Cobi Demmers for excellent technical assistance.

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