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Vegetative Storage Proteins in Poplar[1]

Induction and Characterization of a 32- and a 36-Kilodalton Polypeptide

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ABSTRACT

Bark, wood, and root tissues of several *Populus* species contain a 32- and a 36-kilodalton polypeptide which undergo seasonal fluctuations and are considered to be storage proteins. These two proteins are abundant in winter and not detectable in summer as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodetection. An antibody raised against the 32-kilodalton storage protein of *Populus trichocarpa* (T. & G.) cross-reacts with the 36-kilodalton protein of this species. The synthesis of the 32- and 36-kilodalton proteins can be induced in micropropagated plants by short-day conditions in the growth chamber. These proteins are highly abundant in structural roots, bark, and wood and combined represent >25% of the total soluble proteins in these tissues. Nitrate concentration in the leaves and nitrate uptake rate decreased dramatically when LD plants were transferred to short-day conditions; the protein content in leaves was unaffected. A decrease of the 32- and 36-kilodalton polypeptides occurs after transferring induced plants back to LD conditions. Both polypeptides are glycosylated and can be efficiently purified by affinity chromatography using concanavalin A-Sepharose 4B. The 32- and the 36-kilodalton polypeptides have identical basic isoelectric points and both consist of at least three isoforms. The storage proteins show a loss in apparent molecular mass after deglycosylation with trifluoromethanesulfonic acid. It is concluded that the 32- and 36-kilodalton polypeptides are glycoforms differing only in the extent of glycosylation. The relative molecular mass of the native storage protein was estimated to be 58 kilodalton, using gel filtration. From the molecular mass and the elution pattern it is supposed that the storage protein occurs as a heterodimer composed of one 32- and one 36-kilodalton subunit. Preliminary data suggest the involvement of the phytochrome system in the induction process of the 32- and 36-kilodalton polypeptides.

Most deciduous, woody perennials exhibit a seasonal variation in the nitrogen levels in many of their tissues. The seasonal fluctuation of nitrogen reserves has been reported in several earlier studies (6, 8, 22). During autumnal leaf senescence, much of the leaf nitrogen is translocated into overwintering storage sites, especially the shoot bark. Proteins represent the major fraction of the stored nitrogen and are defined as storage proteins when they are present in large quantities during winter and absent during summer. Specific storage proteins have been identified in the bark of *Malus* (8, 16), *Sambucus* (14), *Robinia* (14), *Acer* (18, 25), *Salix* (25), and *Populus* (4, 20, 25). These proteins accumulate during late summer or early autumn and are highly abundant throughout the winter. It is assumed that with budbreak, the bark storage proteins are degraded into amino acids, which are then translocated to the growing tissues of the trees.

Using SDS-PAGE, van Cleve *et al.* (4) detected a prominent 32-kD polypeptide in the wood of *Populus canadensis* Moench *'robusta'* during the winter that disappeared after budbreak. Sauter and van Cleve (19) demonstrated that the accumulation of protein bodies in the ray parenchymal cells of poplar wood in autumn and their disappearance in spring correlate with the biochemically determined protein content in this tissue. They showed that the protein bodies are the sites of storage of the 32-kD protein. An antibody raised against the 32-kD storage protein of poplar wood also binds specifically to protein bodies in willow wood (18). Wetzel *et al.* (25) reported a 32-kD polypeptide found in the bark of *Populus deltoides*, which made up approximately 30% of the total extractable bark proteins.

Poplars are well suited for breeding programs because of their fast growth, ease of interspecific hybridization, high level of genetic polymorphism, and the simplicity of vegetative and sexual propagation. A recent study (17) showed that poplar is extremely efficient at nitrogen conservation. More than 80% of the whole-tree nitrogen content is conserved during dormancy, and the majority of stored nitrogen can be found in the roots. It might be possible to increase biomass production of poplar through clonal selection for a minimal loss of nitrogen during the winter.

In this paper, we characterize a 32- and a 36-kD storage protein in vegetative tissues of poplar which show seasonal fluctuations and are supposed to occur as glycoforms. We also demonstrate the induction of these two proteins in micropropagated plants under SDs and their disappearance under LDs using a 32-kD antibody. Involvement of the phytochrome system in the induction process is discussed.

MATERIALS AND METHODS

Plant Material

Bark and wood from first-order twigs and bark of structural roots were obtained at different times of the years 1990 and 1991 from 3-year-old *Populus* trees of different clones (*Muhle Larsen, 9/60, Blom: Populus trichocarpa; Donk, Unal: Popu-*

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lus deltoides × *P. trichocarpa; Ghoy: Populus nigra* × *P. deltoides* (= *Populus canadensis*) and *Max 1: Populus nigra* × *P. maximowiczii* growing in the field. These plants were obtained as cuttings from the Institute of Forest Research Station and the Research Institute of Fast Growing Tree Species, Hann. Münden, Germany. Bark of *Salix* sp. was obtained from trees growing in the field. Two *P. trichocarpa* clones (*Muhle Larsen* and *9/60*) were obtained from the Kleinwanzlebener Saatzucht (Einbeck, Germany) as micropropagated plants on agar plates.

Growth Conditions and Treatments

Micropropagated plants (clones *Muhle Larsen, 9/60)* were transferred into hydroponic nutrient solutions in a growth chamber. The nutrient solution was a modified Long Ashton solution with 1.2 mM NO_3^- as the sole nitrogen source. Growth conditions were 22/18°C day/night temperature, 65% RH, and a photon flux of 300 $\mu E\ m^{-2}\ s^{-1}$. Plants were grown for 8 weeks under LD (16:8 h, light:darkness) and were then transferred for 9 weeks to SD (8:16 h, light:darkness). These plants were then transferred back to LD for another 12 weeks. Leaf growth under LD and SD was determined as weekly increases in leaf number.

In another experiment, plants of the clone *Muhle Larsen,* grown for 8 weeks under LD, were transferred to SD for 6 weeks and treated daily with a 15-min red light pulse in the middle of the dark period. Red light was obtained from a Leitz projector combined with a red glass filter ($\lambda = 662$ nm).

Protein Extraction for SDS-PAGE

Bark and wood of first-order twigs and of the main stems were obtained from plants growing in the field and in the growth chamber, respectively. Small pieces (about 200 mg) of leaves, bark, xylem, and structural and fine roots were ground with a mortar and pestle in 5 volumes (v/w) of 100 mM Tris-HCl, 50 mM ascorbic acid, 10 μM leupeptin, 2 mM DTE, pH 8.2. Homogenates were centrifuged in an Eppendorf microcentrifuge (10 min, 10,000*g*) and the supernatants were used for SDS-PAGE after heating (90°C, 10 min) in SDS-sample buffer. Protein in the supernatants was determined according to the method of Bradford (2) using BSA as standard.

Affinity Chromatography

Bark from 3-year-old trees (*P. trichocarpa*, clone *Muhle Larsen*) was collected in November and extracted as described above. Buffer of the protein solution was exchanged with Con A buffer (20 mM Tris-HCl, 0.15 M NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, pH 7.4) using Sephadex G-25 columns. The protein-containing solution (about 1 mg protein) was applied to a Con A-Sepharose 4B column (1 × 5 cm, 14 mg Con A/mL), equilibrated with Con A buffer. The column was washed until no more protein was detectable in the effluent. The bound proteins were eluted with 0.2 M α-methyl glucopyranoside in Con A buffer and the protein concentration in the fractions was determined according to the method of Bradford (2).

Estimation of the Native Molecular Mass

Relative molecular mass of the native storage proteins was determined by gel filtration fast protein liquid chromatography on a Superdex 200 column (Hiload 16/60; Pharmacia, Freiburg, Germany) equilibrated with phosphate buffer (0.1 M NaCl, 0.05 M sodium phosphate, pH 7.2). Bark of *P. trichocarpa* (clone *Muhle Larsen*, collected in November) was extracted as described above. The buffer of the protein solution was exchanged with equilibration buffer using Sephadex G-25 columns. After 600 μg of each standard protein (see below) was added to the protein-containing solution (500 μg of bark proteins/2 mL equilibration buffer), the solution was applied at a flow rate of 1 mL/min, and 0.5-mL fractions were collected. Molecular mass values were obtained by comparing the elution volume of the storage proteins with the elution volume of the following standards of known molecular mass: ferredoxin (440 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (45 kD), chymotrypsinogen A (25 kD), and horse heart Cyt *c* (12.5 kD). For the determination of the elution volume of the storage proteins, each fraction was separated on SDS-PAGE and subsequently silver stained or immunoblotted (see below). The fractions with the highest concentrations of the 32- and 36-kD polypeptides were determined by densitometry of silver-stained gels and by measuring A_{280} of each fraction collected. Both methods gave the same results.

PAGE and Immunoblotting

Crude extracts and purified storage proteins were analyzed by SDS-PAGE as described by Laemmli (11). Slab gels were 0.5 mm thick and consisted of a 5% stacking and a 7 to 20% (linear gradient) separation gel, respectively. Protein solutions were heated for 10 min at 90°C in SDS-sample buffer and subsequently used for SDS-PAGE. High and low molecular weight markers were obtained from Pharmacia.

The subunit composition of the storage proteins was studied using discontinuous linear gradient (4–30%) polyacrylamide gels without SDS in the buffers. Running buffer consisted of 0.09 M Tris, 0.08 M boric acid, and 0.025 M EDTA, pH 8.4. Gels were run for 36 h at 150 V. Following the end of the run, one lane (about 3 mm wide) was excised of the gel (first dimension), equilibrated in SDS-sample buffer, heated at 90°C for 10 min, and then subjected to SDS-PAGE (slab gel 1.5 mm thick) in the second dimension.

The electrophoresed proteins were visualized by silver staining (5) or by immunoblotting as described below. Electrophoretic transfer of polypeptides from SDS-PAGE gels onto 0.2-μm nitrocellulose membrane filters was conducted in 0.035 M Tris, 0.22 M glycine, and 20% methanol in a vertical blotting chamber (530 mA for 2 h, Bio-Rad Trans-Blot cell). Blotted proteins were rendered visible by brief incubation in 0.5% Ponceau S in 1% (v/v) acetic acid. For immunodetection, the nitrocellulose was incubated for 1 h in 5% BSA dissolved in TBS[2] (20 mM Tris-HCl and 500 mM NaCl, pH 7.4). The blots were then incubated for 2 h in TBS, containing antibody (diluted 1:8000) raised against the 32-kD storage protein (see below) and 2% BSA. After the blots were rinsed in TBS, they

[2] Abbreviation: TBS, Tris-buffered saline.

were incubated for 1 h in goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) diluted 1:3333 in TBS with 2% BSA. Rinsed blots were developed for about 1 h in 100 mL of freshly prepared color-developing buffer (100 mM $NaHCO_3$, pH 9.8, and 5 mM $MgCl_2$) containing 1 mL 3% (w/v) nitro blue tetrazolium in 70% dimethylformamide and 1 mL 1.5% (w/v) 5-Br-4-Cl-3-indolyl-phosphate in 100% dimethylformamide. Con A-binding glycoproteins were identified after the proteins were blotted on nitrocellulose. Horseradish peroxidase was coupled to Con A, which had reacted with the immobilized glycoproteins on the blots (3).

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (15) with minor modifications. The first dimension was isoelectric focusing. The measured pH gradient generated by the end of the run was approximately pH 5.0 to 8.5. Ampholines were Servalyt 4 to 9 (Serva). Samples containing about 1 μg of bark proteins, purified by affinity chromatography with Con A, were loaded at the basic end of the focusing gel. After electrofocusing, the gels were placed in SDS-equilibration buffer for 5 min. The second dimension was SDS-PAGE (linear gradient from 7–20% polyacrylamide) on slab gels.

Deglycosylation of Proteins

Deglycosylation of proteins from crude bark extracts (*P. trichocarpa*, clone *Muhle Larsen*, collected in November) with trifluoromethanesulfonic acid was performed on ice for 14 h as described by Karp *et al.* (9) with minor modifications. Deglycosylated samples were washed, in order, with ether:hexane (9:1, v/v), hexane, and 95% (v/v) ethanol. Centrifuged (10,000*g*, 15 min) samples were dried and resuspended in small volumes of SDS-sample buffer for subsequent use.

Antibody Production

The 32-kD storage protein, obtained from bark of 3-year-old trees (*P. trichocarpa*, clone *Muhle Larsen*, collected in November) was purified by preparative SDS-PAGE. SDS-PAGE was conducted using an extended trough-type sample well, 4% stacking gel, and a 7 to 20% gradient separation gel. After electrophoresis, the 32-kD band was rendered visible by brief incubation of the gel in a solution of Coomassie blue (13), and the gel band was excised and rinsed in water. The storage protein was recovered from the minced gel band by electrophoretic elution (7) using the Biotrap system of Schleicher and Schuell (Einbeck, Germany). The eluted 32-kD protein was again electrophoresed and the gel blotted onto nitrocellulose (as described above). After the 32-kD band was stained with 0.5% Ponceau S in 1% (v/v) acetic acid, it was excised, destained in water, and dried. Nitrocellulose sheets containing about 20 μg protein were dissolved in 0.5 mL DMSO and 0.5 mL of Freund's adjuvant complete (Sigma, München, Germany) was added (10). After a preimmune bleed, one female New Zealand white rabbit was injected subcutaneously. Booster injections were given after 2 and 4 weeks and the animals bled 2 weeks later. Preimmune and immune sera were purified by protein A-Sepharose affinity chromatography according to the manufacturer's instructions (Pharmacia).

Nitrate Concentration and Nitrate Uptake

Nitrate concentrations were determined in extracts of leaves prepared according to the method of Bieleski and Turner (1) with minor modifications. Leaves of LDPs and SDPs were ground with a mortar and pestle in 60% (v/v) methanol, 25% (v/v) chloroform, and 15% (v/v) buffer (20 mM Hepes, 5 mM NaF, and 5 mM EGTA). After 12 h at 4°C, the extracts were centrifuged (20 min, 6000*g*). Then, 1 volume of chloroform and 1.5 volumes of buffer were added to 4 volumes of the supernatant. After centrifugation (45 min, 10,000*g*), the upper phase was collected, and an aliquot was used for the enzymatic determination of nitrate (kit from Boehringer, Mannheim, Germany). The nitrate uptake rates for LDPs and SDPs were determined daily, following the depletion of nitrate in the nutrient solution during 24 h. Nitrate was determined enzymatically as described above.

Protein Content

Leaves and bark of LDPs and SDPs were cut in small pieces, ground with a mortar and pestle in 80% methanol, and extracted for 1 h at 70°C. The pellet obtained after centrifugation (10,000*g*, 30 min) was reextracted with 1 N NaOH for 1 h at 80°C and 5 h at 20°C. After centrifugation (10,000*g*, 15 min), protein content in the supernatant was determined according to the method of Bradford (2).

RESULTS AND DISCUSSION

Seasonal Fluctuation of a 32- and a 36-kD Polypeptide

Seasonal variation in the protein pattern of bark tissues of 3-year-old *P. trichocarpa* trees (clone *Muhle Larsen*) was followed by SDS-PAGE and immunoblot analysis. As shown in Figure 1, A and B, two polypeptides with relative molecular masses of 32- and 36-kD started to accumulate in late summer. Their concentrations in the bark tissue increased during autumn and remained at a high level during the winter. In the spring, with budbreak, their level decreased (Fig. 1C), and in the summer they were not detectable. Coomassie-stained gels gave the same results (data not shown). *P. trichocarpa* (clone *Muhle Larsen*) contained 1.3 and 7.3 mg protein/g fresh weight in the bark of first-order twigs in July and January, respectively. During winter, the 32- and 36-kD polypeptides accounted for about 25% of the total soluble bark proteins, as determined by densitometry analysis of silver-stained gels. The 36-kD polypeptide was approximately half as abundant as the 32-kD polypeptide. The high abundance of the 32- and 36-kD polypeptides during winter and their degradation in spring suggest a storage function for both proteins.

Immunoblot analysis shows that the 36-kD polypeptide is immunologically cross-reactive with the antibody raised against the 32-kD polypeptide of *P. trichocarpa* (Fig. 1B and C). The 32- and 36-kD polypeptides do not occur exclusively

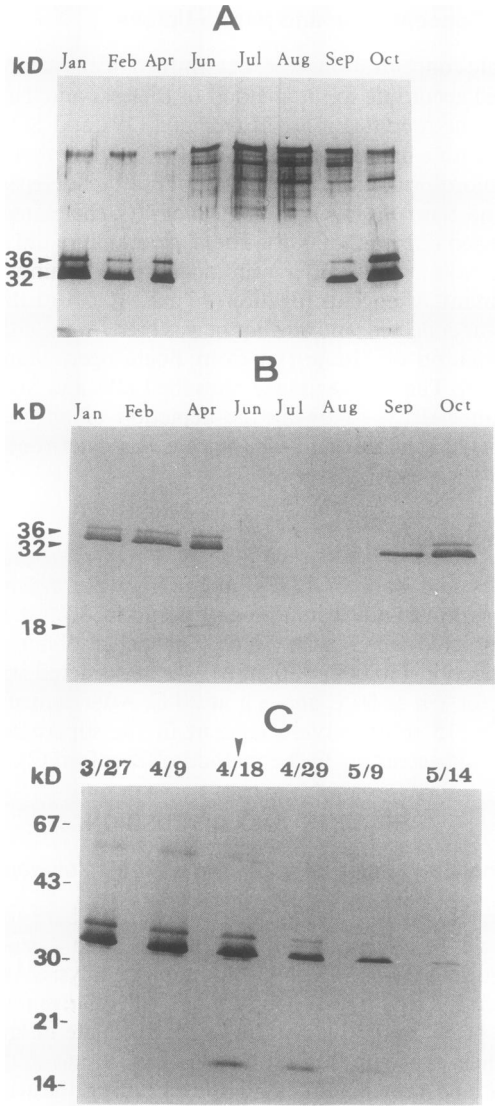

Figure 1. Seasonal variation in the occurrence of the 32- and 36-kD storage proteins in 3-year-old poplar trees. Crude proteins from bark were separated by SDS-PAGE and silver stained or transferred to nitrocellulose. Following transfer, the 32-kD antibody was used as primary antibody. Approximately 2 μg of total protein were loaded in each lane. Molecular mass values in kD are indicated to the left. A, Silver stain; B, immunoblot analysis showing the monthly protein profile of the bark of *P. trichocarpa*, clone *Muhle Larsen*. Letters refer to months of the year from January 1990 to October 1990. C, Immunoblot showing the protein profile of the bark of *P. trichocarpa* (clone *Muhle Larsen*) in spring. Top of the lanes, dates of collection (March 27 to May 14/1991). Arrowhead, time of budbreak.

in the bark of twigs. They are also found in the bark of old structural roots and in wood but never in leaves (Fig. 2). No cross-reactivity was observed even when 20 μg of leaf protein/lane were immunoblotted (data not shown). In winter, the 32- and 36-kD polypeptides occur in several *Populus* species and hybrids between *Populus* species (*P. trichocarpa*, clones: *Muhle Larsen, 9/60, Blom*; *P. deltoides* × *P. trichocarpa*, clones: *Unal, Donk*; *P. deltoides* × *P. nigra*, clone *Ghoy*; *P. nigra* × *P. maximowiczii*, clone *Max 1*; data not shown). Interestingly, in *Salix* sp., only the 32-kD band was detected with the 32-kD antibody of *Populus* (data not shown).

Figure 1C, lanes 3 to 5, shows a low molecular mass protein of about 18 kD, which cross-reacts with the 32-kD antibody. This protein occurred for the first time during budbreak (April 18, 1991, lane 3). Four weeks later the amounts of the 32- and 36-kD polypeptides were dramatically reduced (lane 6) and the 18-kD polypeptide was undetectable. The budbreak and appearance of the 18-kD protein occurred about 4 weeks earlier with clone *9/60* than with *Muhle Larsen,* indicating significant clonal variations (data not shown). It is suggested that the 18-kD protein represents a degradation product of the storage proteins, resulting from proteolytic digestion induced in the spring. The protein with an estimated molecular mass of 58 kD (Fig. 1C, lane 1–3), which cross-reacted with the 32-kD antibody is suggested to represent the heterodimer (32- and 36-kD polypeptide) of the storage protein (see below).

Wetzel *et al.* (25, 26), using SDS-PAGE, showed that a single polypeptide of 32 kD, found in the bark of *P. deltoides*, *P. canadensis*, *Salix spaethii*, and *Salix smithiana*, undergoes seasonal fluctuation and made up approximately 30% of the total extractable bark proteins. Sauter *et al.* (20) and van Cleve *et al.* (4) also detected only one prominent polypeptide of 32 kD in poplar (*P. canadensis* var *robusta*) wood during winter that vanished after budbreak. The 32-kD polypeptide has been considered to be a storage protein (4, 20, 25). Seasonal fluctuation of storage proteins in bark has also been shown to occur in apple (8, 16), maple, willow (25), elderberry, and black locust (14). We showed that the 32-kD polypeptide in *P. trichocarpa* occurred during winter in roots, bark, and

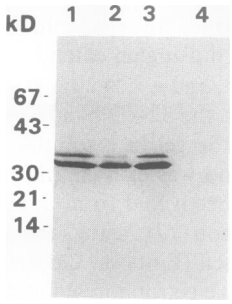

Figure 2. Immunoblot showing the occurrence of the 32- and 36-kD polypeptides in various tissues of 3-year-old poplar trees (*P. trichocarpa*, clone *Muhle Larsen*) in November. Crude proteins were separated on SDS-PAGE and transferred to nitrocellulose. Following transfer, the 32-kD antibody was used as primary antibody. About 2 μg of total protein were loaded in each lane. Molecular mass standards are indicated to the left. Lane 1, Bark of roots; lane 2, bark of first-order twigs; lane 3, wood of first-order twigs; lane 4, leaves.

wood. Therefore, it is very likely that Wetzel *et al.* (25) and van Cleve *et al.* (4), studying the protein pattern of bark and wood tissues of poplars, respectively, examined the same polypeptide. We suggest that the efficient autumn translocation and storage of N in poplar (17) may be due in part to the storage function of the 32- and the 36-kD polypeptide.

It is striking that Wetzel *et al.* (25) and van Cleve *et al.* (4) did not report the seasonal fluctuation of the 36-kD polypeptide that we detected in several *Populus* species, including those examined by these authors. The antibody used by van Cleve *et al.* (4) did not react with a 36-kD polypeptide as our antibody did. Wetzel *et al.* (25) used a borate buffer for extraction of tissue samples, and van Cleve *et al.* (4) extracted oven-dried tissues with Laemmli buffer prior to SDS-PAGE. It is possible that these methods of protein extraction modified the proteins in a way that the 36-kD polypeptide could not be detected in the gels. It is well known, for example, that borate ions can react with neutral sugars (the storage proteins are glycosylated; see below), converting them to charged complexes at alkaline pH. This could influence the protein migration pattern in SDS-PAGE.

Induction of the 32- and 36-kD Polypeptides by SDs

Plants placed under SD conditions maintained a linear increase in leaf number during the first 3 to 5 weeks (data not shown). After about 4 weeks of SD treatment with clone *Muhle Larsen*, leaf expansion and internode elongation stopped, and terminal buds were formed. It must be emphasized that the temperatures were the same under LD and SD conditions (22/18°C, light/darkness). Induction of dormancy in poplar by SD has also been described by Nelson and Dickson (12). When SDPs were transferred back to LD conditions, clone *Muhle Larsen* started with the onset of bud growth after about 8 weeks under LD for dormancy release (data not shown). After 9 weeks under SD conditions, protein content in the bark increased by a factor of 3.7. On the contrary, protein content in leaves was rather unaffected (Fig. 3). The leaves did not show any symptoms of senescence under SD; in contrast, they were darker than under LD conditions. Abscission of leaves did not occur under SD.

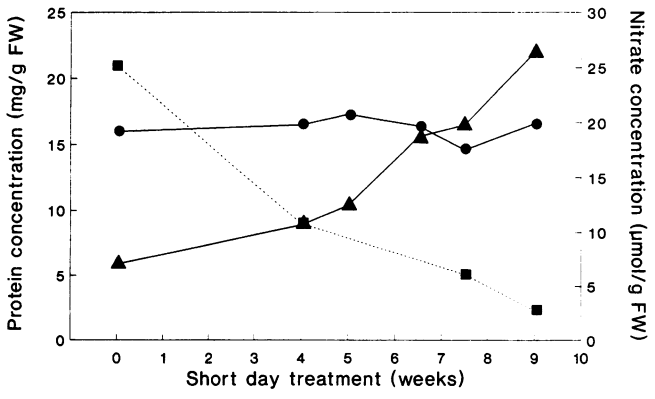

Figure 3. Effect of SD treatment on the protein concentration in bark (▲) and leaves (●) and the nitrate concentration in leaves (■) of *P. trichocarpa* (clone *Muhle Larsen*) growing in a growth chamber. Data are the average of 5 plants. SE <5%; FW, Fresh weight.

To study changes in the nitrogen allocation under SD, nitrate and protein concentrations in SDP and LDP were determined. Nitrate concentration in leaves (Fig. 3) and roots (not illustrated) decreased dramatically under SD conditions (clone *Muhle Larsen*, leaves: 25–2.8 μmol/g fresh weight after 9 weeks of SD treatment), whereas protein content in leaves (Fig. 3) was rather unaffected by the photoperiod. Nitrate uptake rate (average of 24 h) of SDPs was decreased by 67% compared with LDPs (*Muhle Larsen*, 28.8–9.6 μmol/d × g fresh weight). ^{15}N-labeling experiments indicate that the nitrogen for the synthesis of the storage proteins is not derived from degradation of leaf proteins or release of stored nitrate (U. Langheinrich and R. Tischner, unpublished data). We suggest that nitrate, taken up under SD, represents the major nitrogen source for the synthesis of the storage proteins. Although nitrate uptake rates were reduced compared with LD, the amount of nitrogen taken up under SD is sufficient for synthesis of the storage proteins, as calculated from the protein contents and nitrate uptake rates in SDPs. The decrease of the nitrate concentration in leaves of SDPs indicates that nitrate taken up under SD is converted to amino acids rather than stored in the vacuole.

Silver-stained SDS gels (Fig. 4A) and immunoblots (Fig. 4B) clearly show the induction of the synthesis of the 32- and 36-kD polypeptides in the bark of *P. trichocarpa* under SD conditions. After 4 weeks under SD, the 32-kD protein occurred in clone *Muhle Larsen* (Fig. 4B, lane 2). If 20 μg of total bark protein/lane were immunoblotted, the 32-kD protein was detectable for the first time after about 3 weeks under SD (data not shown). The 36-kD polypeptide, present in very small amounts in LDPs (Fig. 4B, lane 1), was detectable in significantly higher amounts after 4 weeks of SD treatment with clone *Muhle Larsen*. Thus, the appearance of the storage proteins is well correlated with the development of terminal buds. Further SD treatment increased the level of the 32- and 36-kD polypeptides, which remained constant after about 7 weeks of SD treatment. The storage proteins also appeared in wood of stems and bark of old roots but not in leaves and fine roots (not illustrated) of induced plants. After 9 weeks under SD, the 32- and 36-kD proteins accounted for about 25% of the total soluble bark proteins (determined by densitometry analysis), which is comparable with the value obtained from bark of trees growing in the field in winter. Therefore, it can be concluded that the increase in protein content in bark under SD conditions is mainly due to an increase in the 32- and 36-kD polypeptides. As in the bark of trees in winter, the 36-kD polypeptide is only half as abundant as the 32-kD polypeptide, as determined by densitometry analysis of silver-stained gels. The faint, sharp protein band with a molecular mass of 31 kD, which cross-reacted with the 32-kD antibody (Fig. 1C, lanes 1–3; Fig. 4B, lanes 5–9) could represent the deglycosylated storage proteins (see below). To our knowledge, the present study shows for the first time an induction of vegetative storage proteins by photoperiod. Staswick (21) reported the induction of vegetative storage proteins in soybean leaves following the removal of pods, the addition of methyl jasmonate, drought stress, and wounding.

When plants of the clone *Muhle Larsen* induced by 9 weeks

under SD were transferred back to LD conditions for 12 weeks, the level of the 32- and 36-kD polypeptides decreased dramatically (Fig. 4B, lane 10). After 8 to 9 weeks of LD (time of budbreak) following SD, the level of the storage proteins decreased, and a protein with a molecular mass of 18 kD, which cross-reacted with the 32-kD antibody, was detectable (Fig. 4B, lanes 8 and 9). This protein is probably identical with the 18-kD protein occurring only in spring (Fig. 1C, lanes 3–5) during the mobilization of the storage proteins (see above). When clone *Muhle Larsen* was treated for 9 weeks with SD and subsequently for 6 weeks with LD, budbreak had not occurred and storage proteins were still abundant in the bark (Fig. 4B, lane 7). Therefore, a strong inverse correlation exists between the occurrence of the storage proteins and shoot growth. It is assumed that during dormancy release in spring or under LD following SD, the storage proteins are degraded and that the released nitrogen is used for the onset of shoot growth.

The photoperiod affects various physiological reactions such as flowering, germination, and growth of internodes. It is known that several of these effects are mediated by phytochrome. Therefore, we wanted to study the involvement of red light effects in the induction process of the storage proteins in poplar. In a preliminary experiment, plants growing under SD conditions were treated with a 15-min red light pulse in the middle of the dark period. Compared with control plants (SD without pulses of red light), red light-treated plants did not develop a terminal bud after 6 weeks of SD treatment, and the levels of the 32- and 36-kD polypeptides were low (Fig. 5). However, the storage proteins were not completely absent in the red light treatment. It could be possible that the red light pulses were not given at the time of highest sensitivity to red light. Therefore, studies under highly reliable conditions are in progress.

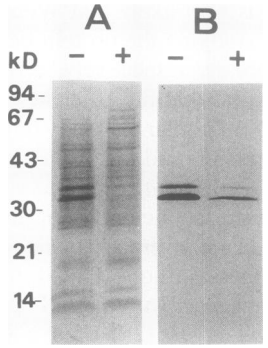

Figure 5. Effect of red light treatment on the appearance of the 32- and 36-kD polypeptides. Approximately 2 μg of crude bark proteins (*P. trichocarpa*, clone *Muhle Larsen*) were separated on SDS-PAGE and silver stained (A) or immunoblotted (B). Molecular mass standards are indicated to the left. −, 6 weeks SD treatment; +, 6 weeks SD treatment with a daily 15-min red light pulse in the middle of the dark period.

Characteristics of the 32- and 36-kD Polypeptides

Crude bark extract was applied to a Con A-Sepharose 4B column, and bound proteins were eluted with α-methyl glucopyranoside. The fraction representing the bound proteins was purified nearly 90% in the 32- and 36-kD polypeptides (Fig. 6, A and B). However, minor amounts of the storage proteins eluted in the unbound fractions (Fig. 6B). Further proof for the glycosylation of the 32- and 36-kD polypeptides is shown in Figure 6C: horseradish peroxidase was bound to Con A, which has reacted with immobilized glycoproteins on nitrocellulose. Color development demonstrated the glycosylation of the 32- and 36-kD polypeptides, which eluted from the Con A-Sepharose column. A control, in which α-methyl glucopyranoside, an inhibitor of the reaction between Con A and glycoproteins, was included in all solutions used for the detection of glycoproteins on blots, gave no signal (not illustrated). Wetzel *et al.* (26) reported the glycosylation of the 32-kD polypeptide in the bark of willow. Van Cleve *et al.* (4) demonstrated that protein bodies of the ray cells were found to be the particular sites of storage of the 32-kD polypeptide. The intravacuolar protein accumulation suggests a pathway

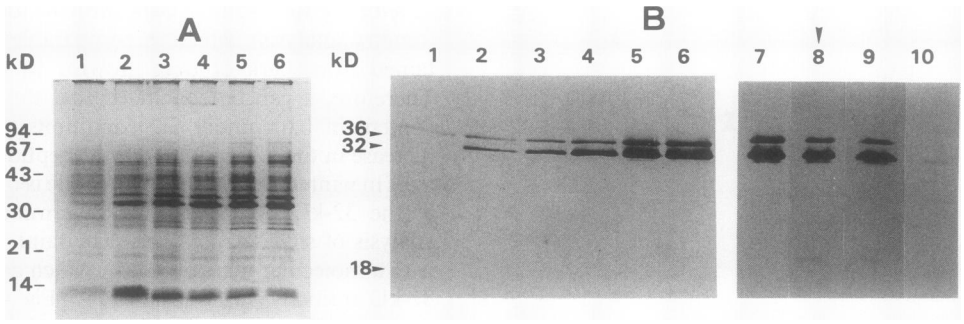

Figure 4. Induction of the synthesis and degradation of the 32- and the 36-kD polypeptides in *P. trichocarpa* (clone *Muhle Larsen*) by SD treatment and LD treatment following SD treatment, respectively. Proteins from crude bark extracts were separated by SDS-PAGE and silver stained (A) or blotted onto nitrocellulose for subsequent immunodetection with a 32-kD antibody (B). Approximately 2 μg of total protein were loaded in each lane. Molecular mass standards in kD are designated to the left. A: lane 1, LD; lane 2, 4 weeks SD, lane 3, 5 weeks SD; lane 4, 6.5 weeks SD; lane 5, 7.5 weeks SD; lane 6, 9 weeks SD; B: lanes 1–6, same as A; lane 7, 9 weeks SD and then 6 weeks LD; lane 8, 9 weeks SD and 8 weeks LD; lane 9, 9 weeks SD and 9 weeks LD; lane 10, 9 weeks SD and 12 weeks LD. Arrowhead, time of budbreak.

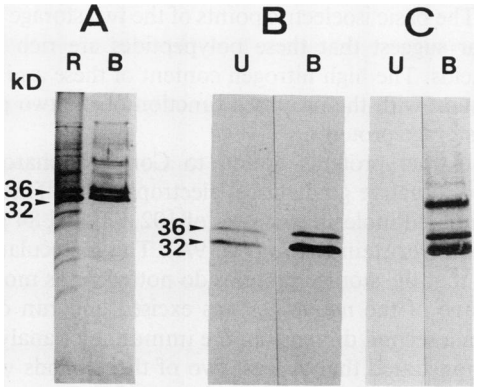

Figure 6. Immunoblot analysis and Con A affinity detection of fractions collected during Con A affinity chromatography of crude bark proteins (*P. trichocarpa*, clone *Muhle Larsen*, collected in November). Proteins of crude extracts (R), unbound proteins (U), and bound proteins (B), which could be eluted from the column by addition of 0.2 M α-methyl glucopyranoside, were separated by SDS-PAGE. Gels were silver stained or transferred to nitrocellulose. Approximately 4 μg of crude proteins and 2 μg of bound and unbound proteins were loaded in the lanes. A, Silver stain of crude bark proteins and bound proteins; B, immunoblot analysis of unbound and bound proteins using the 32-kD antibody; C, Con A affinity detection of glycoproteins in unbound and bound fractions, following blotting of proteins onto nitrocellulose.

of the 32-kD polypeptide via ER and Golgi apparatus. Because many proteins sequestered in the lumen of the ER before being secreted from the cell or transported to other intracellular destinations (such as the Golgi apparatus, vacuoles, plasma membranes, and lysosomes) are glycoproteins, the demonstrated glycosylation of the 32- and 36-kD polypeptides is consistent with their localization in protein bodies.

For further characterization of the two storage proteins, we studied the protein pattern after two-dimensional gel electrophoresis. Bark extract proteins purified by Con A-Sepharose

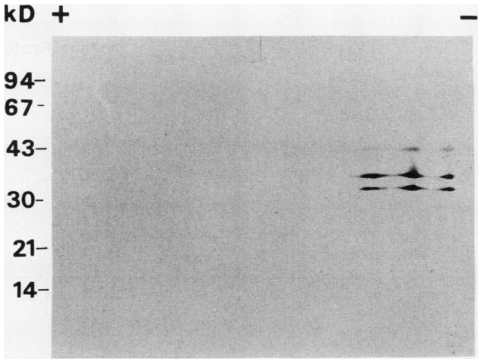

Figure 7. Immunoblot of a two-dimensional gel showing multiple charge forms and glycoforms of the storage proteins of *P. trichocarpa*. + and −, acidic (positive electrode) and basic (negative electrode) directions of the first-dimension gel. Molecular mass standards are indicated to the left. The first-dimension gel was loaded with 1 μg of Con A-Sepharose-purified bark proteins of *P. trichocarpa* (clone *Muhle Larsen*, collected in November).

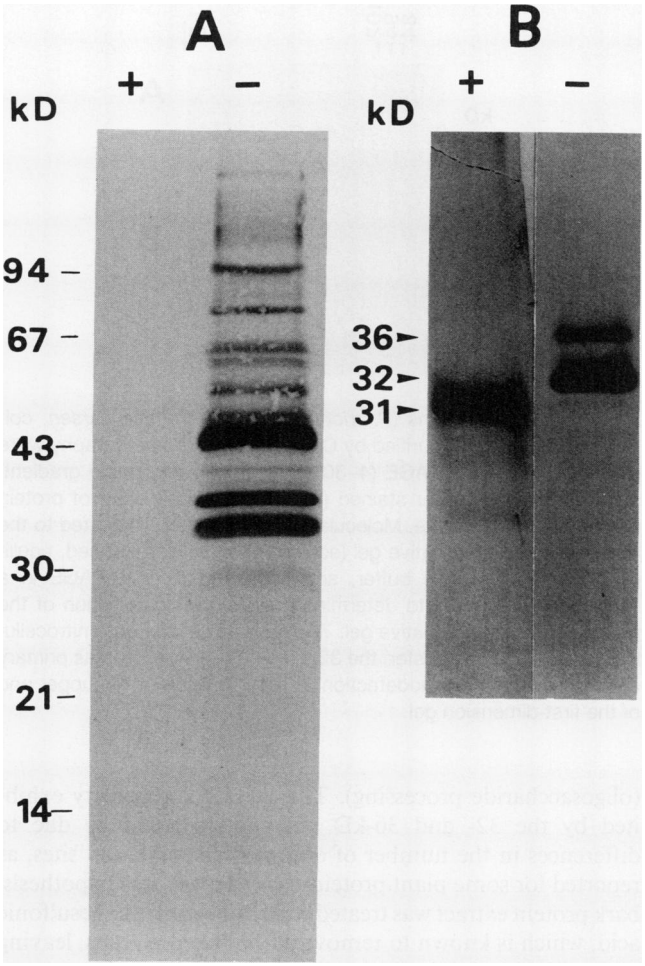

Figure 8. Effect of deglycosylation on proteins from crude bark extracts (*P. trichocarpa*, clone *Muhle Larsen*, collected in November). Untreated (−) or trifluoromethanesulfonic acid-treated (+) bark extracts were separated by SDS-PAGE and blotted onto nitrocellulose. Approximately 2 μg of total protein were loaded in each lane. Molecular mass standards in kD appear to the left. A, Detection of glycoproteins with horseradish peroxidase coupled to Con A; B, immunoblot analysis.

chromatography were separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. The immunoblot analysis (Fig. 7) of the two-dimensional gel showed that each of the 32- and 36-kD polypeptides can be resolved into at least three distinct isoforms. The isoelectric points of the 32- and 36-kD isoforms were identical. The nature of the spots with molecular masses of about 43 kD is not known (artifacts?). From these data and the fact that the 32-kD antibody cross-reacts with the 36-kD polypeptide, we suggest that the 32- and 36-kD polypeptides are very similar in their amino acid sequence. It is known that, in the Golgi lumen and ER, proteins are covalently modified in a variety of ways to produce their final mature forms. In particular, the oligosaccharide chains, previously coupled to proteins in the ER, are extensively modified in the Golgi apparatus by removal and then addition of selected sugar residues

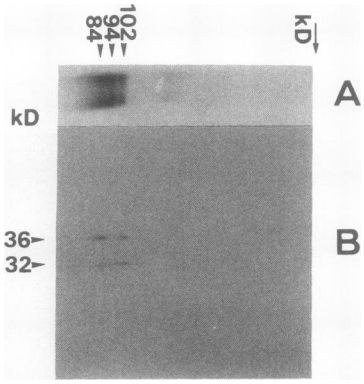

Figure 9. Bark proteins (*P. trichocarpa*, clone *Muhle Larsen*, collected in November), purified by Con A affinity chromatography were separated by native PAGE (4–30%, linear polyacrylamide gradient) and subsequently silver stained (A). Approximately 2 μg of protein were loaded on the lane. Molecular mass values are indicated to the left. One lane of the native gel (equivalent to A) was excised, equilibrated in SDS-sample buffer, and subjected to SDS-PAGE in a second dimension (B) to determine the subunit composition of the proteins visible in the native gel. The gel was blotted onto nitrocellulose, and following transfer, the 32-kD antibody was used as primary antibody for the immunodetection. Arrow, Position of the upper end of the first-dimension gel.

(oligosaccharide processing). The mass heterogeneity exhibited by the 32- and 36-kD polypeptide could be due to differences in the number of occupied glycosylation sites, as reported for some plant proteins (24). To test this hypothesis, bark protein extract was treated with trifluoromethanesulfonic acid, which is known to remove all N-linked glycans, leaving only a GlcNAc residue at the nonreducing end, and to remove also O-linked glycans at lower efficiency (9). In Figure 8A is shown the efficiency of trifluoromethanesulfonic acid in cleaving sugar residues of the 32- and 36-kD polypeptides, because Con A failed to detect glycoproteins after deglycosylation. As expected, the 32- and 36-kD polypeptides showed a loss in apparent molecular mass after deglycosylation, and the antibody detected only one prominent band with a molecular mass of 31 kD (Fig. 8B). Therefore, we conclude that the storage proteins in poplar occur as glycoforms. Thus, the difference in molecular mass between the 32- and 36-kD polypeptides may be due to different extents of glycosylation of the same polypeptide.

According to Voelker *et al.* (24), addition of a single glycan moiety causes approximately a 2-kD increase in protein mass on SDS-PAGE gels. Deglycosylation results in a mass loss of about 5 kD for the 36-kD polypeptide and 1 kD for the 32-kD polypeptide (Fig. 8B). Therefore, we conclude that the glycoforms are due to differences in the number of glycosylation sites occupied. A similar observation was reported by Umbach *et al.* (23), who demonstrated that two proteins in the stigma of *Brassica oleracea* occur as glycoforms, each with several charge forms. The isoelectric points of the glycoforms were identical, as is the case with the 32- and 36-kD polypeptides of *Populus* (Fig. 7). However, it cannot be excluded that the mass heterogeneity exhibited by the storage proteins results from variable processing of carbohydrate groups. The basic isoelectric points of the two storage proteins in poplar suggest that these polypeptides are rich in basic amino acids. The high nitrogen content of these amino acids is consistent with the proposed function of the two polypeptides as storage proteins.

Bark extract proteins bound to Con A-Sepharose were subjected to native gradient gel electrophoresis. Three bands with estimated molecular masses of 102, 94, and 84 kD were visible in silver-stained gels (Fig. 9A). The molecular masses indicate that the storage proteins do not occur as monomers. If one lane of the native gel was excised and run on SDS-PAGE in a second dimension, the immunoblot analysis (Fig. 9B) demonstrated that at least two of those bands visible in the native gel consist of the 32- and 36-kD polypeptide, respectively. This might indicate that the native storage protein exists as isoforms that are composed of subunits of the 32- and 36-kD polypeptide.

The relative molecular mass of the native storage protein using gel filtration was estimated to be 58 kD (Fig. 10). Thus, the discrepancy between the relative molecular mass obtained with native gel electrophoresis and gel filtration is quite high (the anomalous migration of glycoproteins in PAGE and SDS-PAGE is well documented). Interestingly, a protein with a molecular mass of 58 kD, which cross-reacted with the 32-kD antibody, is sometimes observed in immunoblots (*e.g.* Fig. 1C, lanes 1–3) and always when NaCl (0.5 M) was added to the extraction buffer (data not shown). The 32- and 36-kD polypeptides eluted together in a single symmetric peak with their maximal concentrations in the same fraction, as determined by densitometry of silver-stained gels and by measuring A_{280}. Thus, we suggest that the storage protein (extracted from bark of trees in the winter) occurs as a heterodimer composed of one 32- and one 36-kD subunit. Because in LDP (Fig. 4B, lane 1) only the 36-kD protein occurred, it is possible that the subunit composition of the storage protein (in LDP homodi-

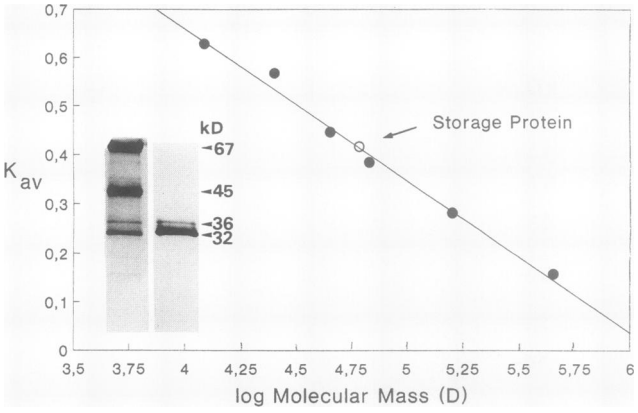

Figure 10. Estimate of the relative molecular mass of the native storage protein using Sephadex 200 gel filtration based on the K_{av} of molecular mass standards (●) and the storage protein (○). (Standard curve correlation coefficient: $r = 0.998$.) Inset: left, silver stain; right, immunoblot analysis of SDS-PAGE separated proteins (about 1 μg protein/lane) of the fraction with the highest concentrations of the 32- and 36-kD polypeptides ($K_{av} = 0.421$). Molecular mass values are indicated to the right.

mer?) is dependent on the stage of development of the plants. Another, not very likely, explanation for the variation in the ratio between the 36- and 32-kD protein could be that the 32-kD protein is an artifact resulting from deglycosylation occurring during the extraction of tissues. The difference between the sum of 32 and 36 kD and 58 kD might result from an anomalous behavior of the glycoproteins in gel filtration and/or SDS-PAGE. It is not known why the 32- and 36-kD polypeptides did not occur in equal amounts in the immunoblots (Figs. 1B, 4B, and 10) or silver-stained SDS gels (Figs. 1A, 4A, and 6A). From SDS-PAGE with and without reducing agents (DTE), we found that the 32- and 36-kD polypeptides are not linked via disulfide bridges (not illustrated). However, it cannot be excluded that the 32- and 36-kD polypeptides are noncovalently linked in the native protein.

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