Plant Physiol. (1995) 108: 141–148

Three Glycosylated Polypeptides Secreted by Several Embryogenic Cell Cultures of Pine Show Highly Specific Serological Affinity to Antibodies Directed against the Wheat Germin Apoprotein Monomer[1]

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Embryogenic tissues of *Pinus caribaea* Morelet var hondurensis produce extracellular proteins; among them germins have been identified. Two-dimensional electrophoresis followed by electroblotting onto a polyvinylidene difluoride membrane allowed isolation and N-terminal amino acid sequencing of extracellular GP111, which is present within the five embryogenic cell lines studied. The amino acid sequence showed strong homologies with the sequences of germins deduced from cDNA sequencing, starting at the same amino acid position but one, compared with other sequences of mature germins deduced from protein sequencing. Immunoblots of embryogenic and nonembryogenic extracellular proteins indicated that the polypeptide GP111 plus two others with similar relative molecular mass values are present in embryogenic cell lines but not in nonembryogenic ones. They were recognized by an antiserum raised against the nonglycosylated monomer of wheat germin. The cross-reaction between pine and wheat apoproteins was highly specific. An antiserum against the glycosylated pentameric germin-like protein (an oxalate oxidase) of barley cross-reacted with all three, as well as with several other glycosylated polypeptides.

Embryogenic tissues of *Pinus caribaea* Morelet var hondurensis consist of stage-1 somatic embryos, i.e. embryos with heads formed of small, closely packed cells attached to long, vacuolated suspensor cells. In contrast to the embryogenic tissue, nonembryogenic cultures consist of small cells with tiny nuclei and thick cell walls.

Recently, we have attempted to characterize the embryogenic state by studying the proteins and glycoproteins ionically bound to the cell surfaces (Domon et al., 1994). The cell surface has been defined by Knox (1992) as everything outside the protoplast. The proteins and glycoproteins extracted by a saline solution (1 M NaCl) from cell cultures were considered "ionically bound" because when cells were washed with water or 0.2 M Glc (as opposed to NaCl) those molecules were detected only as traces in the wash fraction. They were called extracellular but not cell wall proteins because we did not perform experiments on isolated cell walls. Using 2D electrophoresis and affino/immunodetection on western blots, we found highly specific (glyco)protein patterns in the embryogenic lines compared to the nonembryogenic cultures. The major difference is the presence of a set of small glycosylated polypeptides that are strictly representative of the embryogenic lines. We report on the identification of some of these as germin-like proteins.

Germins are a multigene family of glycosylated, water-soluble, homopentameric, protease-resistant proteins. They have been extensively studied by the Lane's group since 1980 (Thompson and Lane, 1980) and later named germin (Grzelczak and Lane, 1984). Their synthesis is concomitant with the onset of growth in germinated wheat embryos. In connection with the nature of association of our pine extracellular proteins with cell surfaces and their relation to germins, it is noteworthy that similar observations led to the conclusion that wheat germins are associated with cell surfaces (Lane et al., 1986). These authors, studying the compartmentation of two forms of germin, compared several wash fractions recovered from a variety of buffers. It is interesting that they found an association of germin with cell surfaces of postimbibition wheat embryos, whereas the protein was not detected in the wash fraction of embryos rinsed with water. This study was critical in establishing that germin, more or less present depending on the stage of growth, was mostly localized at the cell surfaces.

Isoforms of germin have been found during germination in all of the major cereals of economic importance (Lane, 1991; Lane et al., 1991, 1992). Lane et al. (1992) reported that

[1] B.D. was supported by the Bioavenir program funded by Rhône Poulenc with the participation of the Ministère de la Recherche et de l'Espace and of the Ministère de l'Industrie et du Commerce Extérieur.

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Abbreviations: CBB, Coomassie brilliant blue R-250; GP, glycoprotein; G6PDH, Glc-6-P dehydrogenase; NEpHGE, nonequilibrium pH gel electrophoresis; 1D, one-dimensional; 2D, two-dimensional; TTBS, Tris-buffered saline with 0.1% Tween 20.

the germin isoform most conspicuously associated with cell walls is the one that is expressed during germination, and an antigenically related form, pseudogermin, is also associated with the cell walls during embryogenic development. The appearance of germin in the apoplast of germinated embryos (48 h postimbibition) is the major change in the profile of cell wall proteins compared to the apoplast profiles of ungerminated embryos (2 h postimbibition). In cereal monocotyledons, germin expresses oxalate oxidase activity. This was shown for the extensively characterized, naturally occurring, wheat germin oligomer (Lane et al., 1993). The same conclusion was reached by Dumas et al. (1993) after noting that there were sequence homologies between barley oxalate oxidase cDNA and wheat germin sequences reported by Dratewka-Kos et al. (1989).

Although germins are discrete markers of embryo development in cereals (Lane et al., 1992), these proteins are also stress-responsive gene products (Delseny et al., 1994). Two germin-like polypeptides, present in barley roots and immunologically related to wheat germins, have been shown to be associated with salt stress (Hurkman et al., 1991). Recently, the nucleotide sequence of a transcript encoding a germin-like protein has been found in the facultative halophyte *Mesembryanthemum crystallinum* (Michalowski and Bohnert, 1992). As for barley, this protein is root specific.

The data presented here, based on N-terminal amino acid sequence homology, on similarities in mol wts, on similar cell compartmentalization, and on serological relationships, provide evidence for the existence of germins among the set of extracellular proteins characterizing the embryogenic state in *P. caribaea*.

MATERIALS AND METHODS

Source of Plant Material and Maintenance of Cell Culture

Five embryogenic cell lines (E1, E4, E5, E6, and E7) of Caribbean pine (*Pinus caribaea* Morelet var hondurensis) were maintained as embryogenic tissues on proliferation medium as previously described, and one nonembryogenic cell line was cultured on the same medium and used as a control (Domon et al., 1994).

Preparation of Extracellular Proteins from Cell Culture

The extracellular proteins were extracted as previously described (Domon et al., 1994) 9 d after transfer of the cells onto a fresh medium. For precipitation of proteins, 5 volumes of acetone were added to a protein-containing 1 M NaCl solution. After 4 h at 4°C, the protein pellet was recovered by centrifugation (10,000*g*, 10 min, 4°C), washed three times with cold (−20°C) 90% (v/v) acetone in distilled water, pelleted (10,000*g*, 10 min, 4°C), and finally dried in air. Extracellular proteins used for estimation of enzyme activities were precipitated by ammonium sulfate according to Harris (1989).

Gel Electrophoresis

Mini Protean II dual slab cells (Bio-Rad) were used for gel electrophoresis. For SDS-PAGE, acetone extract was suspended in sample buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 0.1 and 2.3% SDS with and without heat treatment (3 min, 95°C) and fractionated by 1D gel electrophoresis (Laemmli, 1970). Slab gels of 10% (w/v) acrylamide were run for 45 min at 20 mA per gel and were analyzed by CBB staining: 1 h of fixation staining with 0.25% (w/v) CBB in 20% ethanol:15% acetic acid (v/v), followed by a destaining step in 10% (v/v) acetic acid, then dried for 2 h unless otherwise stated. For 2D PAGE (NEpHGE-PAGE), first-dimension nonequilibrium pH gradient gel electrophoresis was performed in cylindrical gels (1.5 mm × 80 mm) using 2% (v/v) of Ampholine carrier ampholites, pH 3.5 to 10.0 (Pharmacia). Second-dimension SDS-PAGE was performed in 10% (w/v) polyacrylamide mini slab gels. Fixation, staining, and destaining were as above.

N-Terminal Sequencing

Areas containing spots of interest on the 2D gels were electroblotted onto polyvinylidene difluoride membranes (Applied Biosystems) using the conditions indicated by the supplier. Sequence analysis was performed in the Unité des Eaux et Forêts at the University of Louvain la Neuve (Belgium) using an Applied Biosystems model 477 pulsed liquid phase amino acid sequencer equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer as described by Tao and Kasarda (1989). For GP111 sequence analysis, five blot pieces from the embryogenic line E6 (Domon et al., 1994) were inserted into the reaction chamber for the sequencing run. Searches for homologies with the N-terminal sequence were performed using the peptide sequence data bases SwissProt, Genepept, and PIR using FASTA and BLAST programs.

Production of Antibodies Directed against the Barley Oxalate Oxidase and the Wheat Germin Produced in *Escherichia coli*

The preparation of antibodies against the barley oxalate oxidase was described previously (Dumas et al., 1993). The wheat germin was produced in *E. coli* by cloning the sequence coding for mature germin in the same translational reading frame as the *malE* gene, which encodes the maltose-binding protein (Dumas et al., 1993) in the pMAL-cRI vector (New England Biolabs). A protein extract was prepared from a 400-mL culture by sonication and the maltose-binding protein-germin fusion protein was purified by affinity chromatography on an amylose column (New England Biolabs) according to the manufacturer's instructions. The purified fusion protein (400 μg) was administered to a rabbit in four intramuscular injections. Ten days after the last boost, the serum was collected, clarified by centrifugation, and stored at −20°C.

Western Blots

Electrotransfer of proteins onto a 0.45-μm nitrocellulose membrane (Schleicher & Schuell) was performed as described by Burnette (1981), except that ethanol replaced methanol in the transfer buffer, in a Bio-Rad blotting ap-

paratus for at least 2 h at constant voltage (100 V) at 4°C. The efficiency of blotting was controlled by the presence of Bio-Rad prestained molecular standards on the membrane. After transfer to nitrocellulose, sheets were fixed with 10% acetic acid:25% isopropanol.

For immunostaining, the free binding sites on nitrocellulose membranes were blocked with 3% (w/v) gelatin at 25°C in Tris-buffered saline and treated essentially as described by Faye et al. (1993) with immuneserum diluted 1/1000 in 1% (w/v) gelatin in TTBS followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1/2000 in 1% (w/v) gelatin in TTBS and washed, successively, in TTBS and 0.2 M diethanolamine.

Enzyme Assays

Oxalate oxidase activity was assayed by a modified procedure of Sugiura et al. (1979). Protein extracts were incubated in succinate buffer (45 mM, pH 3.8) containing 1 unit mL^{-1} horseradish peroxidase, 3-methyl-2-benzo-thiazolinone hydrazone (1.5 mM), and *N,N*-dimethylaniline (1.5 mM). The assay produces 1 μmol of indamine dye per μmol of oxalate converted.

For detection of oxalate oxidase activity after gel electrophoresis, the proteins were loaded without heat denaturation on SDS-PAGE gels (10% [w/v] polyacrylamide) and transferred onto a nitrocellulose sheet, which was then directly incubated with oxalic acid (2.5 mM) in succinate buffer, pH 4.0 (25 mM succinic acid and 3.5 mM EDTA) containing 1 unit mL^{-1} horseradish peroxidase and 4-chloro-1-naphthol (0.6 mg mL^{-1}) as the staining reagent (Dumas et al., 1993).

G6PDH (EC 1.1.1.49) activity was tested as a marker for cytoplasmic proteins. Total proteins were extracted according to Peleman et al. (1989) or extracellular proteins were assayed using G6PDH reagent and a protocol from Sigma Diagnostics (catalog No. 345–1).

Protein concentrations were determined according to Bradford (1976) using the Bio-Rad protein assay kit with BSA as standard.

Image Analysis

No fewer than three CBB-stained 1D SDS-PAGE and NEpHGE gels or blots were created for each experiment, and were scanned and analyzed using the freeware program NIH image 141 provided by Dr. Wayne Rasbaud (National Institutes of Health, Research Services Branch, Bethesda, MD).

RESULTS

GP111, an Extracellular Glycosylated Polypeptide of the Embryogenic Cell Cultures of Caribbean Pine

In agreement with our previous results (Domon et al., 1994), the five embryogenic cell cultures studied (E1, E4, E5, E6, and E7) give characteristic 2D patterns of extracellular proteins. The majority of the spots are representative of the embryogenic profile, such as spots 94, 103, and 111 on Figure 1. A few others, such as 52, 54, 55, 57, and 75, are

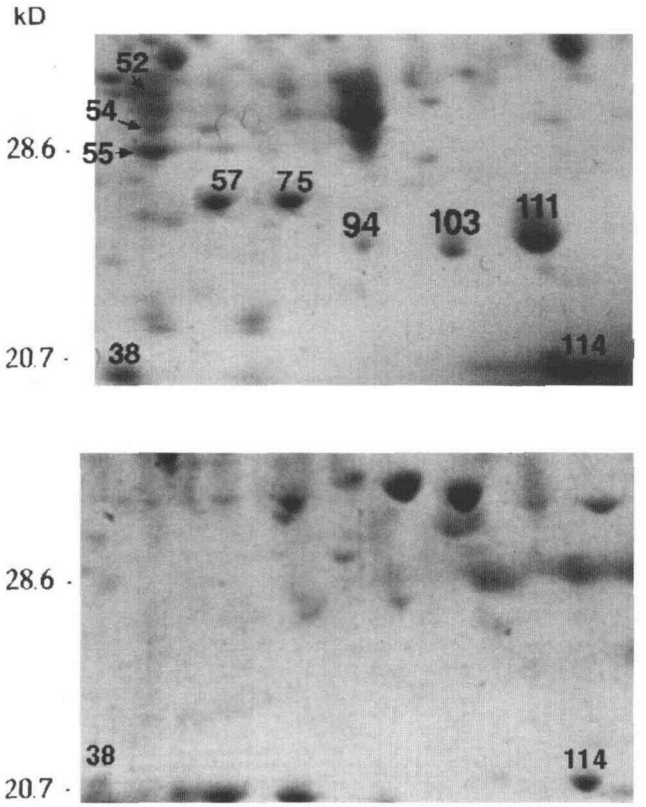

Figure 1. Partial NEpHGE-PAGE electrophoretogram of extracellular polypeptides of embryogenic cell line E6 (upper) and nonembryogenic line NE7 (lower). Spots 94, 103, and 111 are representative of the pine embryogenic profiles; spots 52, 54, 55, 57, and 75 are cell-line specific; and spots 38 and 114 are present in embryogenic and nonembryogenic cultures (Domon et al., 1994).

associated with the phenotype (early or late stage 1) of the somatic embryos. The corresponding spots are absent from the 2D patterns of nonembryogenic cultures, showing that they are specific for embryogenic cultures. Conversely, among the other spots, 38 and 114 are present in the two types of cultures (embryogenic and nonembryogenic) (Fig. 1).

The cytoplasmic marker enzyme G6PDH was used to assess the presence or absence of contaminating cytoplasmic proteins (Boudet et al., 1981). G6PDH was not detected in the extracellular extracts, although activities measured in total protein extracts were around 4 pkat mg^{-1} (fresh weight) and 7 pkat mg^{-1} for embryogenic and nonembryogenic cultures, respectively.

Of the embryogenesis-specific polypeptides we chose to purify GP111, which is relatively abundant, is strongly representative of the embryogenic profiles, and migrates well away from the other spots. The embryogenic line E6, with a typically polarized phenotype (Domon et al., 1994), was selected.

The N-Terminal Sequence of Pine Protein GP111 Shows Strong Similarity to the Corresponding Sequence in Mature Wheat Germin

Figure 2 shows the multiple alignments of the amino acid sequences of germin and germin-like proteins deduced

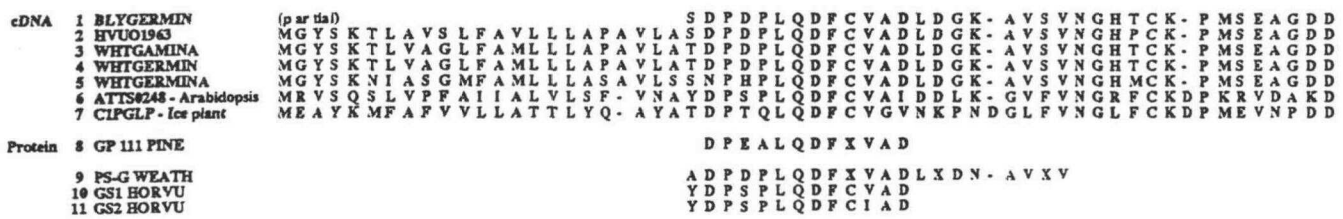

Figure 2. Alignment of the N-terminal amino acid sequence of pine GP111 with several amino acid sequences of germin precursors and mature germins. The N-terminal amino acid sequence (12 residues) for the pine GP111 (embryogenic line E6) was determined by direct amino acid sequencing. The germin precursors sequences 1 to 7 were obtained from GenBank (1, No. L15737, Lane et al., 1993; 2, No. U01963, Hurkman et al., 1994; 3, No. J04592, Dratewka-Kos et al., 1989; 4, No. M63223, Lane et al, 1991; 5, No. M63224, Lane et al., 1991; 6, No. Z17674, M. Raynal, F. Grellet, M. Laudié, Y. Meyer, R. Cooke, and M. Delseny, unpublished data, from the *A. thaliana* ATTS0248 transcribed sequence described by Höfte et al., 1993; 7, No. M93041, Michalowski and Bohnert, 1992). The sequence for mature germin 9, No. S27247, was from PIR (Lane et al., 1992), and the sequences for mature germin 10, No. P28525, and 11, No. P28526, were from SwissProt (Hurkman et al., 1991).

from cDNA sequencing (Nos. 1–5 are coding elements for cereal germins; No. 6 is for a germin precursor isolated from immature siliques of *Arabidopsis thaliana*, and No. 7 is for a germin-like protein from unstressed roots of the halophyte *Mesembryanthemum crystallinum*) with those of pine GP111 (No. 8) and cereal germins (Nos. 9–11) deduced from microsequencing of N-terminal regions of the mature proteins. The pine protein GP111 shows strong similarity to corresponding N-terminal sequences in wheat germins and to open reading frames in germin-like coding elements isolated from other angiosperms (cereals, *M. crystallinum*, *A. thaliana*).

When comparing the N-terminal sequences deduced from microsequencing, it appears that the mature protein starts at the same site, except one amino acid is absent. One major difference between these two groups of plants (cereals and pine) could be the site of excision of the signal peptide on the germin precursor, with, as a consequence, one amino acid less in the N-terminal part of the mature pine protein. Pine sequence No. 8 is not a consensus sequence for several embryogenic lines but, rather, was obtained for a single pine protein (GP111) from cell line E6. The consensus sequence for the multiple alignments in Figure 2 is DPXXLQDFCVAD.

Serological Cross-Reactivities with the Polyclonal Immunesera against the Germin-Like Oxalate Oxidase of Barley

Antibodies against homogeneous, naturally glycosylated oxalate oxidase from barley seedlings (Boehringer Mannheim, EC 1.2.3.4, specific activity approximately 5 units mg^{-1} protein) were prepared by Dumas et al. (1993) and were used to probe western blots after 1D or 2D separation of the extracellular proteins from embryogenic and nonembryogenic cell cultures. It has been reported previously that preheating (2 min at 100°C) the germin-like protein extracts allows the denaturation of germin oligomers (125–130 kD) to monomers (25–26 kD) (Gzelczak and Lane, 1983; Dumas et al., 1993; Lane et al., 1993). Under our conditions, when heat pretreatment was omitted the 1D and 2D extracellular protein profiles were unchanged regardless of the SDS concentration (0.1 or 2.3%) used in the sample buffer. The use of 0.1% SDS allows the activity of cereal oxalate oxidase enzyme to be revealed after blotting onto a nitrocellulose sheet (Dumas et al., 1993; Lane et al., 1993). Therefore, for subsequent affino-blotting, pine protein samples have been used without the heat step and with 0.1% SDS in the buffer. The 1D analyses were performed on five embryogenic and one nonembryogenic cell lines. Purified oxalate oxidase (Boehringer Mannheim) was used as a control.

As shown on the western blots in Figure 3, these antibodies recognize several bands. Among them a discrete band is detected around M_r 25,000 in the embryogenic lines E1, E4, E5, E6, and E7, along with the band corresponding to the protomeric form of barley oxalate oxidase. Under our conditions, this enzyme has been shown to be partially denatured without heating in 0.1% SDS; however, most of the labeling is around 125 kD, a value that conforms with the oligomer mass of wheat germin (Lane et al., 1993).

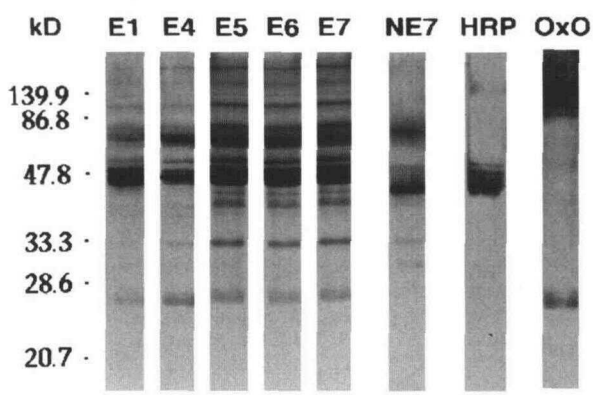

Figure 3. Cross-reaction of extracellular polypeptides of embryogenic (E1, E4, E5, E6, and E7) and nonembryogenic (NE7) cultures of Caribbean pine with barley anti-oxalate oxidase antibodies, after SDS-PAGE. Horseradish peroxidase (HRP, Sigma) and oxalate oxidase (OxO, Boehringer) were used as controls. Five micrograms of protein were loaded on each lane. After SDS-PAGE, proteins were electrotransferred and detected on nitrocellulose (0.45 μm) membranes. Prestained estimated molecular mass standards from Bio-Rad were phosphorylase B (139.9 kD), BSA (86.8 kD), ovalbumin (47.8 kD), carbonic anhydrase (33.3 kD), soybean trypsin inhibitor (28.6 kD), and lysozyme (20.7 kD).

In addition, these antibodies also recognize several other bands. Lane et al. (1992) raised polyclonal rabbit antibodies against wheat germin to detect germin and pseudogermin among soluble embryo proteins as well as for immunocytological localization of the proteins in cell walls. A 1/4000 dilution allowed the selective detection of a doublet of germin glycoforms on blots, although those proteins were not detected by Coomassie blue staining. Their antiserum was much more reactive with the wheat germin apoprotein than with its N-glycans or the same N-glycans of other wheat proteins. On the other hand, the anti-oxalate oxidase antibodies that we have used for immunoblotting, raised against the glycosylated oligomeric form of the enzyme, were likely much more heavily directed toward N-glycans, although a nonglycosylated fusion protein including the 21-kD mature germin monomer was recognized by the antibodies (Dumas et al., 1993). In our experiments, barley-oxalate oxidase antiserum used at 1/1000 dilution was more reactive toward N-glycans than it was toward the pine "germin-like" proteins, as shown by the numerous N-linked glycans detected in our extracellular (glyco)protein samples (Fig. 3). As an example, we show that horseradish peroxidase (Boehringer Mannheim, EC 1.11.1.7., specific activity 250 units mg^{-1} protein), probably through its covalently attached N-glycans, cross-reacts with the anti-oxalate oxidase antibodies. At the same M_r values, we note (Fig. 3) the presence of particularly sharp bands in the embryogenic lines.

This is also illustrated by Figure 4, which shows western blots of 2D gels of extracellular proteins of one embryogenic cell line of pine after immunodetection with the same antiserum. Numerous spots along with important smears characteristic of the presence of associated glycans are detected on the blot. As expected from our published results (Domon et al., 1994), cross-reaction occurs primarily at M_r values above 30,000 in E7, whereas it is interesting to note that the three polypeptides on which we focused our attention (GP94, GP103, and GP111) are heavily stained (Fig. 4, stars).

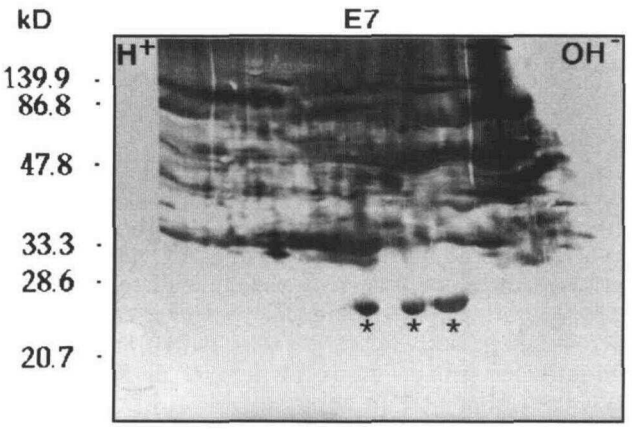

Figure 4. Immunoreactivity of the extracellular polypeptides of an embryogenic line (E7) with barley anti-oxalate oxidase antibodies, after 2D NEpHGE electrophoresis. Stars indicate, from right to left, the polypeptides GP111, GP103, and GP94. Thirty micrograms of protein were loaded onto the cylindrical gels. Electrotransfer, detection, and molecular mass standards are as in Figure 3.

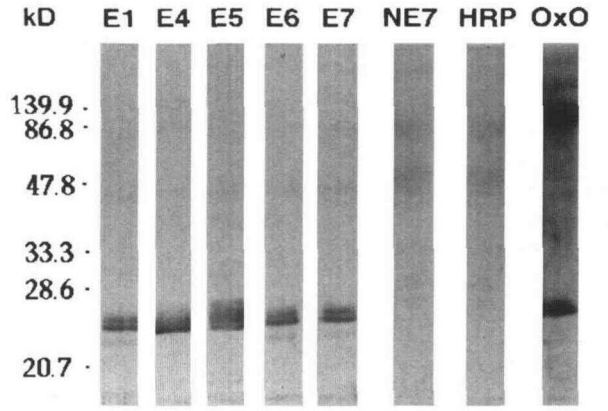

Figure 5. Analysis of extracellular pine proteins after SDS-PAGE with antibodies directed against the wheat germin monomer expressed in *E. coli*. Five micrograms of protein were loaded onto each lane. Electrotransfer, detection, molecular mass standards, and controls are as in Figure 3.

Immunological Relationships between the Wheat Germin Monomer Expressed in *E. coli* and Pine Polypeptides, Including GP111, That Were Isolated from Embryogenic Cell Lines

Polyclonal antibodies raised against a bacterially expressed wheat germin apoprotein monomer (Dumas et al., 1993) were used to show highly specific serological identity with antibodies obtained from a transgenic protein expressed in its monomeric form and devoid of N-glycans.

As shown in Figure 5, proteins are detected by immunoblotting with antigermin monomer antiserum. Among the embryogenic lines (E), only one area, showing an apparent molecular mass of 25 to 27 kD, cross-reacts with the antibodies. A closer observation of these blots shows that, depending on the embryogenic lines, slightly different zones cross-react with this highly specific probe. Indeed, doublets of germin-like monomers are clearly detected in E6 and E7, whereas in E1, E4, and E5 a slightly larger area is covered by the positive signal. On the contrary, no signal is observed in the nonembryogenic line (NE7). Oxalate oxidase (OxO) and horseradish peroxidase (HRP) were used as positive and negative controls, respectively. The lane loaded with purified oxalate oxidase displays the two characteristic heavily stained bands, showing partial denaturation without pretreatment in the presence of 0.1% SDS and confirming the recognition of the monomer as well as the oligomer by the antibodies. As expected, horseradish peroxidase did not cross-react. The cumulative amounts of extracellular pine proteins applied to the 1D gels (Fig. 5) were identical to those of purified oxalate oxidase and horseradish peroxidase. The antigenically positive bands in lanes E1 through E7 contain small amounts of protein that were only weakly CBB stained in the protein patterns observed before electroblot transfer from the SDS-PAGE gel. Unexpectedly, the antigenicity of the pine protein ap-

pears to be high; this could reflect high amino acid sequence homologies between pine and wheat germins.

To confirm that the labeling with antibodies against nonglycosylated wheat germin monomer was restricted to the extracellular proteins of the embryogenic cell lines, we treated the blots of intracellular proteins with the same antibodies. As expected, antigenicity was not observed for any intracellular proteins (data not shown). Moreover, some embryogenic tissues were also washed with water and the corresponding blots were treated with the same probe. This simple device was used to confirm, by their absence from water washes, that the bands of interest are associated with cell surfaces. The affino-detection on 2D western blots of extracellular proteins of the embryogenic tissues (E7 as an example is shown in Fig. 6) strengthens the results presented in Figure 5. Indeed, the two glycopeptides GP111 (accounting for 2% of the extracellular protein extracts, which represent 6% of the total proteins) and GP103, and to a lesser extent GP94, cross-react with antibodies against the wheat-germin apoprotein. For our western blot experiments the same extracts were always run simultaneously. One was later used for electrotransfer and the other was dye stained (Coomassie blue). The three spots showing cross-reactivity with the antiserum occupy identical positions and have been designated as spots 111, 103, and 94. As shown in Figure 5, no signal is observed on the 2D blots of nonembryogenic line 7 (data not shown).

These results demonstrate the significant and essential relationship between these three GPs and the monomer of the germin isoform made by germinated wheat.

This gives evidence for the presence of germin-like proteins in the extracellular compartment of the embryogenic tissues (somatic embryos at stage 1 of development) of Caribbean pine. The cross-reaction of three glycopeptides on the 2D blots leads us to speculate about the presence of glycoforms in this family of proteins. The presence of glycoforms is not surprising, since Jaikaran et al. (1990), investigating the types of glycans present on wheat germins, showed that glycosylation can be prevented by tunicamycin and that the N-glycans have different sugar terminal residues according to the isoform of germin, G or G'; the isoform G contains antennary GlcNAc but G' does not.

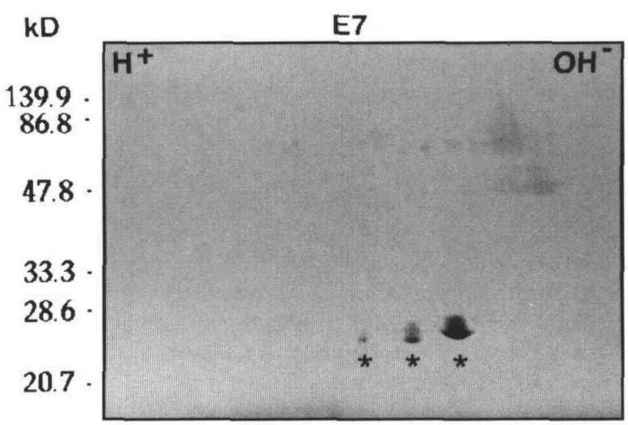

Figure 6. Serological relationships between the wheat germin monomer expressed in *E. coli* and the extracellular polypeptides GP111, GP103, and GP94 (stars from right to left) of the pine embryogenic line E7 after 2D NEpHGE. Thirty micrograms of protein were loaded onto the cylindrical gels. Electrotransfer, detection, and molecular mass standards are as in Figure 3.

Oxalate Oxidase Activity

Lane et al. (1993) demonstrated that wheat germin expressed oxalate oxidase activity, and Dumas et al. (1993) showed that oxalate oxidase synthesized by barley grains was a germin. As already published by both groups, in the case of cereals it is possible to assess directly the oxalate oxidase activity of a protein sample on a gel or blot, and the sensitivity of the method is very high. In spite of numerous attempts, we have not been able to detect any enzymatic activity of this kind in our samples of pine protein, either by assaying oxalate oxidase activity in solution following the procedure of Sugiura et al. (1979) or by detection of the activity on a blot. The absence of detectable activity in unfractionated preparations of proteins could result from the small amount of the pine enzyme (the germin monomers accounting for less than 0.2.% of the total proteins). The absence of oxalate oxidase activity on the blot could also be due to our extraction and separation conditions not being compatible with the recovery of the enzyme's active form. SDS (0.1%) is present in the sample and electrophoretic buffers, and any enzyme activity of the pine protein may not withstand SDS denaturation. Indeed, any oligomer that is present is completely converted to monomer when pine samples are in the presence of 0.1% SDS. It is interesting that oxalate oxidase activity has never been observed in gel blots or solution assays of natural or transgenic monomers, and the stability of germin oligomers is, in fact, related to their role in oxalate degradation (Lane, 1994). We also suggest that the germin-like proteins detected that associated exclusively with the cell walls of the somatic embryos of pine play a role closer to that of pseudogermin than to that of germins, even if they express the same immunologically related properties as germin.

DISCUSSION

Germins, widely studied by Lane and co-workers (for reviews, see Lane, 1991, 1994) and more recently by Dumas et al. (1993), are among the best-characterized protein markers of cereal-embryo development. Germin itself first accumulates in the walls of germinated wheat embryos during seedling development, whereas the pseudogermin isoform first accumulates in the walls of mature wheat embryos during embryogenic development (see Lane et al., 1992). Germin precursors are also present in other angiosperm species, such as the model plant *A. thaliana*. The germin clone isolated from this species by Höfte et al. (1994) was from a cDNA library established from mRNAs of immature siliques of different developmental stages.

Our findings provide evidence for the presence of germin-like proteins among the extracellular GPs of the embryogenic tissues of *P. caribaea* cultured on solid medium. Our results are based on similarities with mature germins or germin-like precursors of the other plants, i.e. their

presence in the extracellular compartment, the M_r of their glycosylated monomers (25,000–27,000), a consensus sequence in their N termini, and overall cross-reactivity against antibodies to mature wheat germin apoprotein.

The function of germin-like proteins within the embryogenic tissues of pine is unknown. But their presence, associated with cell surfaces of stage-1 somatic embryos and not with nonembryogenic callus, might be closely related to the typically polarized structure of the somatic embryos of conifers (Tautorus et al., 1991). Somatic embryos consist of an embryonic region with dense cytoplasmic cells and a suspensor with prolonged, highly vacuolated cells, although the suspensor is not so precisely organized as in zygotic embryos (Hakman and Fowke, 1987). We have indirect evidence that the suspensor cells are derived from the embryo itself (Lainé et al., 1992). Only small embryonal cells can survive cryopreservation treatment of pine embryogenic tissues, and, during regrowth, new suspensor cells are produced and embryonic clusters develop. Somatic embryos can mature, germinate, and produce shoots and roots in a manner similar to that of unfrozen embryogenic tissues. Due to tremendous cell enlargement and vacuolation, the cytoplasm that is still highly active becomes restricted to a thin peripheral layer (Hakman et al., 1987). Thus, newly formed suspensor cells are capable of water uptake leading to enormous expansion. The likely roles for germin in the vacuolation and terminal differentiation of ground tissues described by Lane (1994) are compatible with the observations on the conifer embryogenic system, where both processes, initiation and termination of wall expansion, occur when the embryogenic tissues are maintained on the proliferation medium. It is interesting that the "germin-like" GPs (GP111, GP103, and GP94) are steadily synthesized by our pine embryogenic lines during a 2-week period between the two subcultures (J.M. Domon, A. David, and H. David, unpublished data). The presence of germins within stage-1 somatic embryos and not in nonembryogenic callus makes these extracellular glycoproteins the first markers of somatic embryogenesis in this species of pine. They might represent a new group of proteins that can be used for diagnosis of embryogenesis not only in this group of trees but, due to similarities with other immature-embryo-related germins, also in several other in vitro systems. The question of the possible physiological implications of such markers is raised along with their specific cell localization.

ACKNOWLEDGMENTS

We wish to thank Dr. J.M. Dupuis for his action in initiating the collaboration between H.D. and B.D., and also Professor M. Briquet (Faculté des Sciences Agronomiques, Université Catholique de Louvain, Louvain-la-Neuve, Belgium) for assistance in microsequencing.

Received October 28, 1994; accepted January 17, 1995.

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