Plant Physiol. (1995) 108: 1369–1377

Expression of a Zeatin-*O*-Glucoside-Degrading β-Glucosidase in *Brassica napus*[1]

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A β-glucosidase was purified from seeds of *Brassica napus* L. (oilseed rape). The 130-kD native enzyme consisted of a disulfide-linked dimer of 64-kD monomers. Internal amino acid sequences were used to construct degenerate primers for polymerase chain reaction-mediated cloning of cDNA for the enzyme. One nearly full-length and one partial β-glucosidase-encoding cDNA clone were isolated and sequenced. Southern hybridization showed that β-glucosidase is encoded by a small gene family in *B. napus*. Northern hybridization showed that the genes are expressed in the seed, with a low degree of expression in other tissues. In the seed, the expression started at 30 days after pollination (DAP), with the highest expression at 40 DAP. The size of the transcript was approximately 1900 nucleotides. In situ hybridization to developing seeds of *B. napus* showed that the β-glucosidase expression started at 30 DAP around the provascular tissue in the embryo axis. In the cotyledons, mRNA initially accumulated around the provascular tissues but was detected first at 35 DAP. At 40 DAP, expression occurred in most parts of the seed. In situ hybridization also detected β-glucosidase mRNA in shoots, young roots, and the basal part of the hypocotyls. Zeatin-*O*-glucoside was identified as a natural substrate for *B. napus* β-glucosidase.

Plant hormone concentration in vivo is regulated not only by de novo synthesis but also by activation and inactivation mechanisms. One mode of inactivation is by glucosylation, and with the exception of ethylene, all plant hormones have been identified as glucosyl conjugates (Cohen and Bandurski, 1982; Letham and Palni, 1983; Schneider and Schmidt, 1990). In the case of the cytokinins, there are two major forms of glucosylation: *N*-linked and *O*-linked. The cytokinin-*N*-glucosides are regarded as irreversible inactivation products, whereas the cytokinin-*O*-glucosides are probably temporarily inactivated products from which the cytokinins may be released by the action of specific glucosidases. However, the phytopathogen *Agrobacterium rhizogenes* contains a β-glucosidase encoded by the *rolC* gene, which can release free cytokinin from both cytokinin-*N*- and -*O*-glucosides (Estruch et al., 1991). The ability of this enzyme to also hydrolyze the *N*-glucosides is probably the reason for the ability of the pathogen to promote abnormal plant growth.

Information on plant-derived plant hormone glucoside-degrading β-glucosidases is scarce. Brzobohaty et al. (1993) isolated a zeatin-*O*-glucoside-degrading β-glucosidase from maize. However, since the main physiological substrates of this enzyme are the hydroxamic acid glucosides in maize (Babcock and Esen, 1994), its involvement in plant hormone metabolism is far from clear. In *Brassica napus*, zeatin-*O*-glucoside has been identified in many parts of the plant (Singh and Sawhney, 1992), although no glucosidase responsible for its hydrolysis has been isolated. We have investigated the zeatin-*O*-glucoside-degrading β-glucosidases in *B. napus* and have purified an enzyme with this activity. cDNA clones encoding the enzyme have been sequenced and the expression pattern of the corresponding genes has been studied by northern and in situ hybridizations.

MATERIALS AND METHODS

Plant Material

Seeds of the *Brassica napus* cultivars Hanna and the dihaploid line 20516 K of Svalöfs Karat were obtained from Svalöf Weibull AB (Svalöv, Sweden). Hanna was used in all protein extractions, whereas RNA and DNA were isolated from 20516 K. Plants were grown in a greenhouse with 8-/16-h dark/light conditions to obtain seeds for RNA hybridizations. Young shoots for in situ hybridization were taken from 14-d-old plants grown in a greenhouse. Seeds were germinated in Petri dishes on moistened filter paper in an incubator with 8-/16-h dark/light conditions at 24°C to produce seedlings for in situ hybridization. The seedlings were 4 d old at time of harvest. *Arabidopsis thaliana* cv Landsberg *erecta* was used to extract genomic DNA for Southern hybridization.

Source of Chemicals

Zeatin, kaempferol, sinigrin, cellobiose, methyl-α-D-mannopyranoside and *p*-nitrophenyl-β-D-glucopyranoside were obtained from Sigma. Zeatin-*O*-glucoside was obtained from Apex Organics (Leicester, UK). Kaempferol-3-*O*-glucoside was isolated from pine (*Pinus silvestris* L.)

[1] This research was supported by grants from the Swedish Research Council for Forestry and Agriculture.

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Abbreviations: DAP, days after pollination; SSC, saline sodium citrate (0.15 M NaCl, 0.015 M Na-citrate, pH 7.0).

needles and was a gift from Dr. Lennart Lundgren (Department of Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden).

Enzyme Purification

A modification of the glycerol-based method of Begbie (1979), originally used for purification of seed protein bodies, was used for enzyme purification. *B. napus* seeds (600 g) were crushed in 300 mL of glycerol per 100 g of seeds in a Waring Blendor. The homogenate was squeezed through a 52-μm nylon mesh and centrifuged at 55,000*g* for 2 h. The fat cake on top of the extract was discarded when the clear glycerol extract was removed from the tubes. The glycerol extract was thereafter dialyzed against 3 × 20 L of 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, for 48 h at 4°C, and applied to a column of lentil-lectin-coupled Sepharose. The bound proteins were eluted with 0.2 M methyl-α-D-mannopyranoside in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl. The eluate was applied to a Mono Q 10/10 column equilibrated with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, using a fast protein liquid chromatography chromatograph (Pharmacia Biosystems, Uppsala, Sweden). The column was eluted with a 0 to 1 M NaCl gradient. The β-glucosidase activity eluted at 150 mM NaCl. Fractions containing β-glucosidase were combined and further purified by gel chromatography on a Sephacryl S-300 column, 100 × 1.7 cm, equilibrated with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl. The β-glucosidase-containing fractions were identified by measuring enzymatic activity. Fractions were tested for the presence of contaminating myrosinases by western analysis with the monoclonal anti-myrosinase antibody 3D7 (Lenman et al., 1990).

Enzyme Assays

β-Glucosidase activity was measured spectrophotometrically using the chromogenic substance *p*-nitrophenyl-β-D-glucopyranoside. To 50 μL of undiluted purified enzyme was added 450 μL of 5 mM *p*-nitrophenyl-β-D-glucopyranoside in 20 mM KH_2PO_4, pH 6.5. The mixture was incubated at 37°C, and the reaction was terminated by the addition of 500 μL of 2 M Na_2CO_3. The liberated *p*-nitrophenol was measured at 410 nm. An extinction coefficient of 17.5 $cm^2/\mu mol$ was used for calculations of specific activity (John and Schmidt, 1985).

The purified β-glucosidase was tested for myrosinase and cellobiase activities. To 50 μL of undiluted enzyme was added 450 μL of 20 μM sinigrin or 1 mM cellobiose in 50 mM Na-acetate, pH 4.5. The reaction mixture was incubated at 37°C for 24 h. Enzymatic activity was determined by measuring Glc liberated from the hydrolysis of sinigrin or cellobiose with the Glc-oxidase method Merckotest (Merck, Darmstadt, Germany).

The purified β-glucosidase was incubated with zeatin-*O*-glucoside or kaempferol-3-*O*-glucoside. To 50 μL of undiluted enzyme was added 250 μL of 20 μM zeatin-*O*-glucoside or kaempferol-3-*O*-glucoside in 20 mM KH_2PO_4, pH 6.5. Reactions were incubated at 37°C for 24 h. The reactions were stopped by addition of 300 μL of cold acetone. The samples were then incubated on ice for 20 min and centrifuged at 12,000*g* for 20 min. The supernatants were evaporated to dryness, and the zeatin-*O*-glucoside sample was dissolved in 0.2 M acetic acid-triethylamine, pH 4.8, whereas the kaempferol-3-*O*-glucoside sample was dissolved in 0.1% TFA, 20% methanol. The samples were applied to a Sephasil C_8 column in a SMART chromatography system (Pharmacia Biosystems) and eluted with linear gradients of 0 to 50% methanol in 0.2 M acetic acid-triethylamine buffer (zeatin-*O*-glucoside) or 20 to 100% methanol in 0.1% TFA (kaempferol-3-*O*-glucoside). The hydrolysis of the glucosides to the corresponding aglycones was determined by the appearance of zeatin or kaempferol in the HPLC chromatograms. Samples of pure zeatin, zeatin-*O*-glucoside, kaempferol, and kaempferol-3-*O*-glucoside were run separately on the column for calibration.

PAGE and Western Blotting

SDS-PAGE gels with an acrylamide concentration of 10% were prepared and run as described by Dobberstein et al. (1979). Protein samples were reduced with 25 mM DTT, heat denatured at 95°C for 5 min, and alkylated by adding 5 μL of 0.5 M iodoacetamide. Unreduced protein samples were prealkylated with 5 μL of 0.5 M iodoacetamide. Western blotting with the anti-myrosinase antibody 3D7 was performed as described by Towbin et al. (1979).

Deglycosylation of β-Glucosidase

Purified β-glucosidase was deglycosylated with peptide-*N*-glycosidase F and *O*-glycosidase (Boehringer Mannheim). To 1 μg of purified β-glucosidase (in a 10-μL volume of 10 mM Tris-HCl, pH 8.0, 50 mM NaCl) was added 10 μL of 0.01% SDS, and after heating at 95°C for 3 min, 1 μL of 0.1% Nonidet P-40 was added. For digestion with peptide-*N*-glycosidase F, 5 μL of 0.5 M Na-phosphate, pH 7.1, was added. For digestion with *O*-glycosidase, 5 μL of 0.5 M Na-acetate, pH 5.2, was added. Water was added to a final volume of 50 μL and the mixture was once again heated at 95°C for 3 min. One milliunit of deglycosylating enzyme (1 unit hydrolyzes 1 μmol of glycopeptide per min) and 0.25 μL of 100 mM PMSF were added, and the reaction mixture was incubated overnight at 37°C. The mobility of the deglycosylated β-glucosidase in SDS-PAGE was compared with that of nondeglycosylated β-glucosidase run on the same gel.

Amino-Terminal Protein Sequence Determination, Protein Digestion, and Isolation and Sequencing of Peptides

Purified protein was reduced and alkylated as described (Tempst et al., 1990), separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane for the determination of the amino-terminal sequence. Purified protein was digested with endoproteinase Lys C as described (Falk et al., 1995b), and the resulting peptides were reduced and alkylated (Falk et al., 1995b) and separated on a μRPC C_2/C_{18} column in a SMART chromatography system (Pharmacia Biosystems) using a 5 to 60% gradient of acetonitrile in 0.1% TFA. Peptide and amino-terminal se-

quences were determined using Applied Biosystems 470A and 476A sequencers and the PROFTA program (Applied Biosystems).

PCR Reactions, Screening of cDNA Library, and Sequencing of DNA

The peptide sequences HWDMPA and CEDKVN from the β-glucosidase were used to construct the degenerate primers AGTGGATCCCA(C/T)TGGGA(C/T)ATGCC(A/C/G/T)GC (forward primer) and AGTGGATCC(G/A)TT(A/C/G/T)AC(T/C)TT(G/A)TC(T/C)TC (reverse primer) for PCR. The first nine nucleotides at the 5′ ends were added to facilitate cloning of the PCR product. PCR was carried out using a cDNA library, corresponding to mRNA in developing *B. napus* seeds, as the template. The construction of this cDNA library has been described (Ellerström et al., 1992). The first three cycles of the PCR reaction were carried out at 37°C to facilitate binding of the degenerate primers to homologous sequences. Primer concentration was 6 μM of each primer mixture. The $MgCl_2$ concentration was 5 mM, the amount of cDNA library used for the reaction was 40 ng, corresponding to approximately 10 ng of cDNA, the final reaction volume was 50 μL, and a total of 38 cycles was run. All other conditions were as described by Ausubel et al. (1994). The amplified fragment was purified from the reaction mixture in agarose gels and labeled with [α-^{32}P]dCTP using random primers and Klenow enzyme for the synthesis of labeled strands. The labeled fragment was used as a probe for colony screening (Sambrook et al., 1989) of the same cDNA library from which it was amplified. cDNA clones were subcloned into pBluescript II SK+ and sequenced using the 373A DNA sequencing system (Applied Biosystems).

Southern and Northern Hybridizations

Southern blots with 10 to 20 μg of restriction-enzyme-digested genomic DNA from the dihaploid line 20516 K were as described by Rödin et al. (1993). Isolation of total RNA and northern blotting with the Hybond C Extra membrane (Amersham) were performed as described (Falk et al., 1992). A fragment of the β-glucosidase cDNA clone corresponding to bp 975 to 1206 was labeled with [α-^{32}P]dCTP and used as a probe in the hybridizations. The membranes were hybridized in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na-citrate), 5× Denhardt's solution, 0.5% SDS, 0.2 mg/mL salmon sperm DNA at 65°C. Southern hybridizations were washed twice for 5 min each time in 2× SSC, 0.1% SDS at room temperature, and twice for 15 min in 0.7× SSC, 0.1% SDS at 58°C. Northern hybridizations were washed twice for 5 min in 2× SSC, 0.1% SDS at room temperature, and twice for 15 min in 0.1× SSC, 0.1% SDS at 50°C. The hybridizations were analyzed using the PhosphorImager from Molecular Dynamics (Sunnyvale, CA).

In Situ Hybridization

Antisense and sense β-glucosidase probes corresponding to bp 975 to 1206 were labeled with [α-^{33}P]-UTP by in vitro transcription, essentially as described in the Maxiscript protocol (Ambion, Austin, TX). Unincorporated nucleotides were removed by two precipitations with ammonium acetate. Plant tissues were fixed in 4% paraformaldehyde, 100 mM Na-phosphate, pH 7.0, and 0.25% glutaraldehyde for 6 to 24 h at room temperature and then dehydrated in a graded series of ethanol solutions, followed by a xylene incubation before infiltration and embedding in Histowax (Histolab, Göteborg, Sweden). Sections were cut 5 to 6 μm thick with a Microm M330 microtome (Heidelberg, Germany) and mounted on aminopropyltriethoxy silane-treated slides. Pretreatment of sections was essentially as described by Cox and Goldberg (1988), but the treatment with BSA was omitted. Sections were treated with 2 μg/mL proteinase K and 2 mg/mL Gly in 200 mM Na-phosphate, pH 7.0, at 37°C for 30 min. Hybridizations and washes were according to Cox and Goldberg (1988) with minor modifications. The final wash was in 0.1× SSC at 55°C for 1 h.

RESULTS

Purification of a β-Glucosidase from *B. napus*

Preliminary studies on myrosinase and β-glucosidase in *B. napus* indicated that the enzymes behaved similarly during ion exchange and gel chromatography. However, centrifugation of glycerol extracts of *B. napus* seeds accomplished a good separation of the β-glucosidase from the myrosinases. The myrosinases were detected in the fat cake on top of the glycerol, whereas the β-glucosidase was dissolved in the glycerol. The centrifugation of seed glycerol extracts was therefore adopted as an efficient means to separate the two enzymes from each other. The residual contaminating myrosinases were detected in the flow-through fraction in the subsequent lentil-lectin affinity chromatography, and could thus be discarded. Lentil-lectin affinity chromatography was used because β-glucosidase was expected to be a glycoprotein in a dicotyledon like *B. napus* (Esen, 1993). The eluate from the lentil-lectin column was concentrated by ion exchange chromatography before application to a gel chromatography column. β-Glucosidase was essentially pure after the gel chromatography. Two minor contaminating proteins of 36 and 38 kD, possibly binding to β-glucosidase, did, however, appear in the fractions, but no myrosinases could be detected. β-Glucosidase was eluted as a broad asymmetric peak during the gel chromatography. This may be due to the existence of β-glucosidase complexes with a broad molecular mass range. Such complexes have been noted for β-glucosidases in *Hevea* (Selmar et al., 1987) and in maize (Esen and Cokmus, 1990). The purification scheme is summarized in Table I and Figure 1. As can be seen in Table I, only a small fraction of the total β-glucosidase activity in the extract was isolated with this method. The major portion of the remaining activity in the glycerol extract was probably due to a small contamination of myrosinases, which have a higher specific activity against the artificial substrate *p*-nitrophenyl-β-D-glucopyranoside. The presence of myrosinase activity in the starting material also explains the seemingly low purification factor obtained.

Table I. *Purification of a β-glucosidase from B. napus seeds*
For details of purification, see "Materials and Methods." pNP, *p*-Nitrophenol.

Purification Step	Total Protein	Total Activity	Specific Activity
	mg	*nmol pNP min^{-1}*	*nmol pNP min^{-1} mg^{-1}*
Dialyzed glycerol extract from 600 g of seeds	387	774	2
Lentil lectin Sepharose	5.1	15.3	3
FPLC Mono Q pH 8.0	5.1	15.3	3
Sephacryl S-300	1.5	12	8

Characterization of a β-Glucosidase in *B. napus*

The β-glucosidase was analyzed by SDS-PAGE without prior reduction and gave rise to a band corresponding to a molecular mass of 130 kD (data not shown). In contrast, after prior reduction and alkylation, a single band of 64 kD was noted, which showed that the protein consisted of two disulfide-linked subunits. Deglycosylation of the enzyme with peptide-*N*-glycosidase F reduced the apparent size of the enzyme to approximately 60 kD as estimated by SDS-PAGE (data not shown), showing the existence of one or more *N*-linked carbohydrate side chain(s), which is also indicated by the affinity of the enzyme for lentil lectin. No mobility shift in SDS-PAGE was detected after treatment of the enzyme with *O*-glycosidase.

The K_m of the β-glucosidase preparation for *p*-nitrophenyl-β-D-glucopyranoside was 155 μM with a specific activity of 8 nmol min^{-1} mg^{-1}. A significant activity toward zeatin-*O*-glucoside was found (specific activity 0.2 nmol min^{-1} mg^{-1}). The enzyme did not hydrolyze cellobiose, kaempferol-3-*O*-glucoside, or the myrosinase substrate sinigrin.

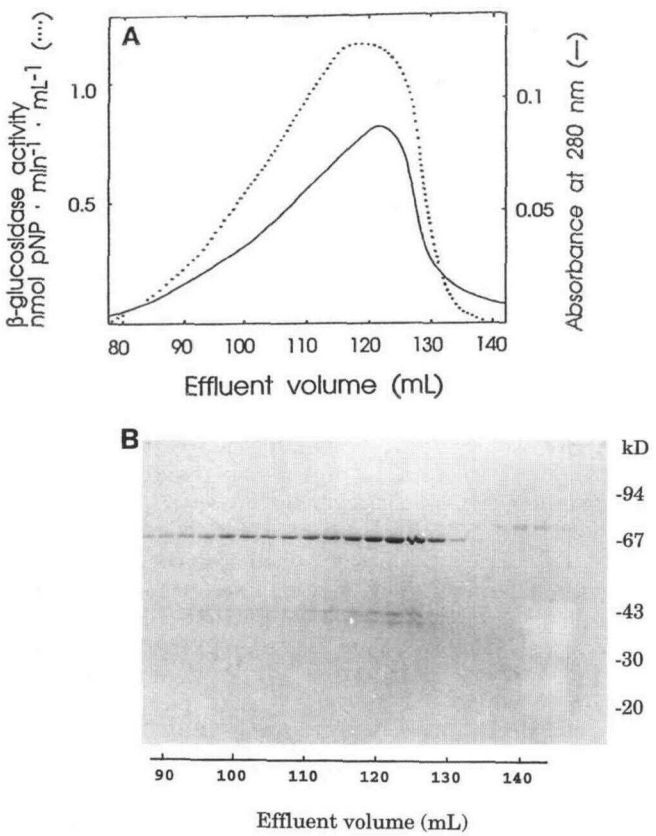

Figure 1. A, Gel chromatography of a partially purified β-glucosidase preparation from *B. napus* on a Sephacryl S-300 column with a void volume of 77 mL and a total volume of 220 mL. B, SDS-PAGE of fractions from the chromatography in A stained with Coomassie brilliant blue.

Sequence of a β-Glucosidase-Encoding cDNA Clone from *B. napus*

The isolated β-glucosidase was digested with Lys C protease, and the resulting peptides were reduced and alkylated with 4-vinylpyridine. The peptides were then separated by reversed phase chromatography. Four peptides and the intact protein were subjected to amino acid sequence determination, and amino acid residues could unequivocally be identified in 113 positions. Two short sequence segments were chosen as guidance for synthesis of degenerate oligonucleotides for use as PCR primers. Since the peptide sequences displayed homology to other β-glucosidases, their relative location in the protein sequence could be assumed and only one primer pair had to be synthesized. PCR amplification using as a template a

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-21  MKFPLLGLLLLVTLVGSPTRAEEGPVCPKTETLSRASFPEGFMFGTATAS
                    +1
 30  YQVEGAVNEGCRGPSLWDIYTKKFPHRVKNHNADVAVDFYHRFREDIKLM
 80  KKLNTDALRLSIAWPRIFPHGRMEKGNSKEGVQFYHDLIDELLKNDLTPL
130  VTIFHWDMPADLEDEYGGFLSERVVPDFVEYANFTFHEYGDKVKNWITFN
                                *
180  EPWVFSRSAYDVGKKAPGRCSPYIKDFGHLCQDGRSGFEAYVVSHNLLVS
230  HAEAVDAFRKCEKCKGDKIGIAHSPAWFEPEDVEGGQRTVDRVLDFIMGW
280  HLDPTTYGDYPQSMKDAVGARLPKFTKAQKAKLKGSADFVGINYYSSFYA
330  KASEKPDYRQPSWATDSLVEFEPKTVDGSVKIGSQPSTAKMAVYAAGLRK
380  LVKYIKDRYGNPEIIITENGYGEDLGEKDTDHSVALNDHNRKYYHQRHLL
430  SLHQAICEDKVNVTSYFVWSLMDNFEWLDGYTARFGLYYIDFQNNLTRME
                  *                                    *
480  KESATCSLNSSNRA
                *
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Figure 2. Deduced amino acid sequence of β-glucosidase clone Bgl4:1 from *B. napus*. "+1" indicates the start of the mature protein. Sequences that have been verified by amino acid sequencing are underlined. Potential sites for *N*-linked glycosylation are indicated by asterisks.

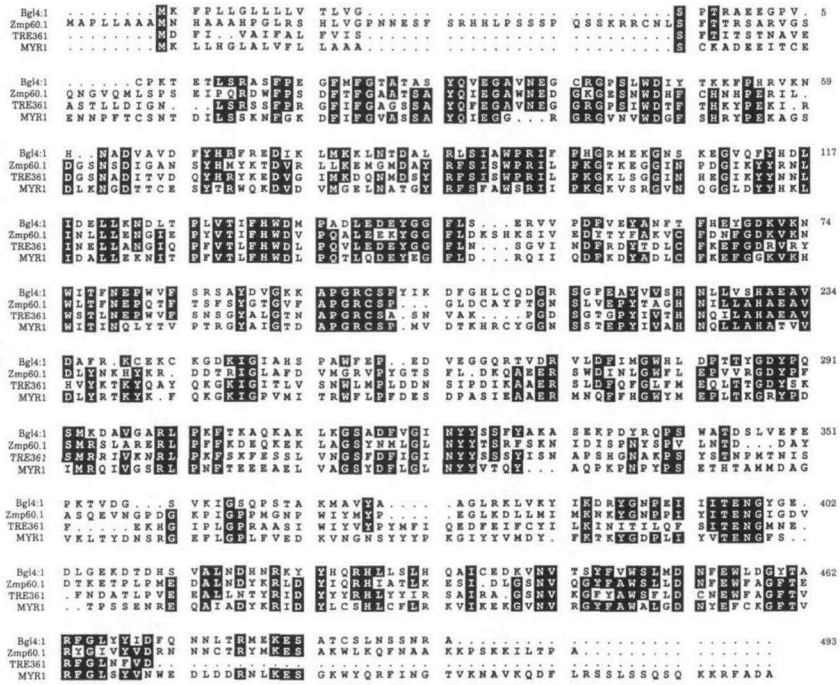

Figure 3. Alignment of deduced amino acid sequences from cDNA clones encoding plant β-glucosidases. Amino acid residues shared by more than two of the sequences are shaded. The deduced amino acid sequence of *B. napus* β-glucosidase was numbered from the start of the mature protein. The sequences are: β-glucosidase from *B. napus*, Bgl4:1; β-glucosidase from *Zea mays*, Zmp60.1 (Brzobohaty et al., 1993); cyanogenic β-glucosidase (linamarase) from *Trifolium repens*, TRE361 (Oxtoby et al., 1991); and β-thioglucosidase (myrosinase) from *B. napus*, MYR1 (Falk et al., 1992).

cDNA library made with *B. napus* seed mRNA produced a fragment of approximately 900 bp. This fragment was used as a probe to screen the cDNA library. Two clones, Bgl4:1 and Bgl2:1, were chosen for characterization. Bgl4:1 contained the coding information for the β-glucosidase, but lacked 122 bp of the 3′ untranslated sequence. These were present in the shorter Bgl2:1 clone, which otherwise was completely identical to Bgl4:1 in the overlapping 708 bp.

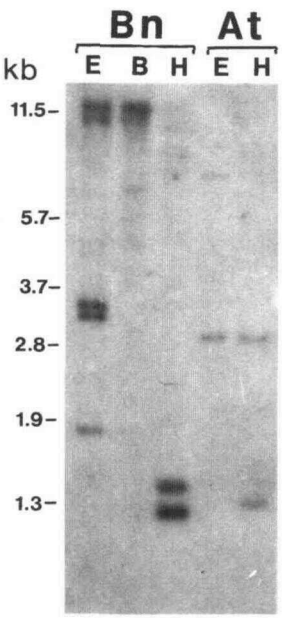

Figure 4. Southern analysis using a probe specific for *B. napus* β-glucosidase. Genomic *B. napus* (Bn) and *A. thaliana* (At) DNA were digested with *Eco*RI (E), *Bam*HI (B), or *Hin*dIII (H).

The amino-terminal 21 amino acids of the deduced amino acid sequence of Bgl4:1 (Fig. 2) had the characteristics of a signal peptide, and the signal peptidase cleavage site was assumed to be adjacent to the determined amino terminus of the mature enzyme. Other β-glucosidases have been shown to be cleaved at a homologous position (Falk et al., 1992). The mature protein consisted of 493 amino acid residues, corresponding to a molecular weight of approximately 56,000, a value slightly lower but still consistent with the apparent molecular weight of 60,000 estimated for the deglycosylated protein by SDS-PAGE. The sequence contained four potential sites for *N*-linked glycosylation (noted by asterisks in Fig. 2). At least one of these, Asn^{441}, seems to be used, since this residue could not be identified by amino acid sequence determination. The other three potential glycosylation sites have not been analyzed by amino acid sequence determination.

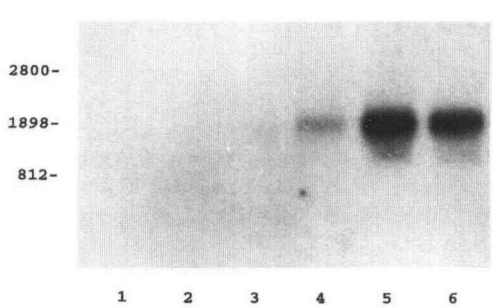

Figure 5. Northern analysis of total RNA from developing seeds of *B. napus*, using a probe specific for *B. napus* β-glucosidase. Lane 1, Seeds at 20 DAP; lane 2, seeds at 25 DAP; lane 3, seeds at 30 DAP; lane 4, seeds at 35 DAP; lane 5, seeds at 40 DAP; and lane 6, seeds at 45 DAP. Marker indicates size of RNA in nucleotides.

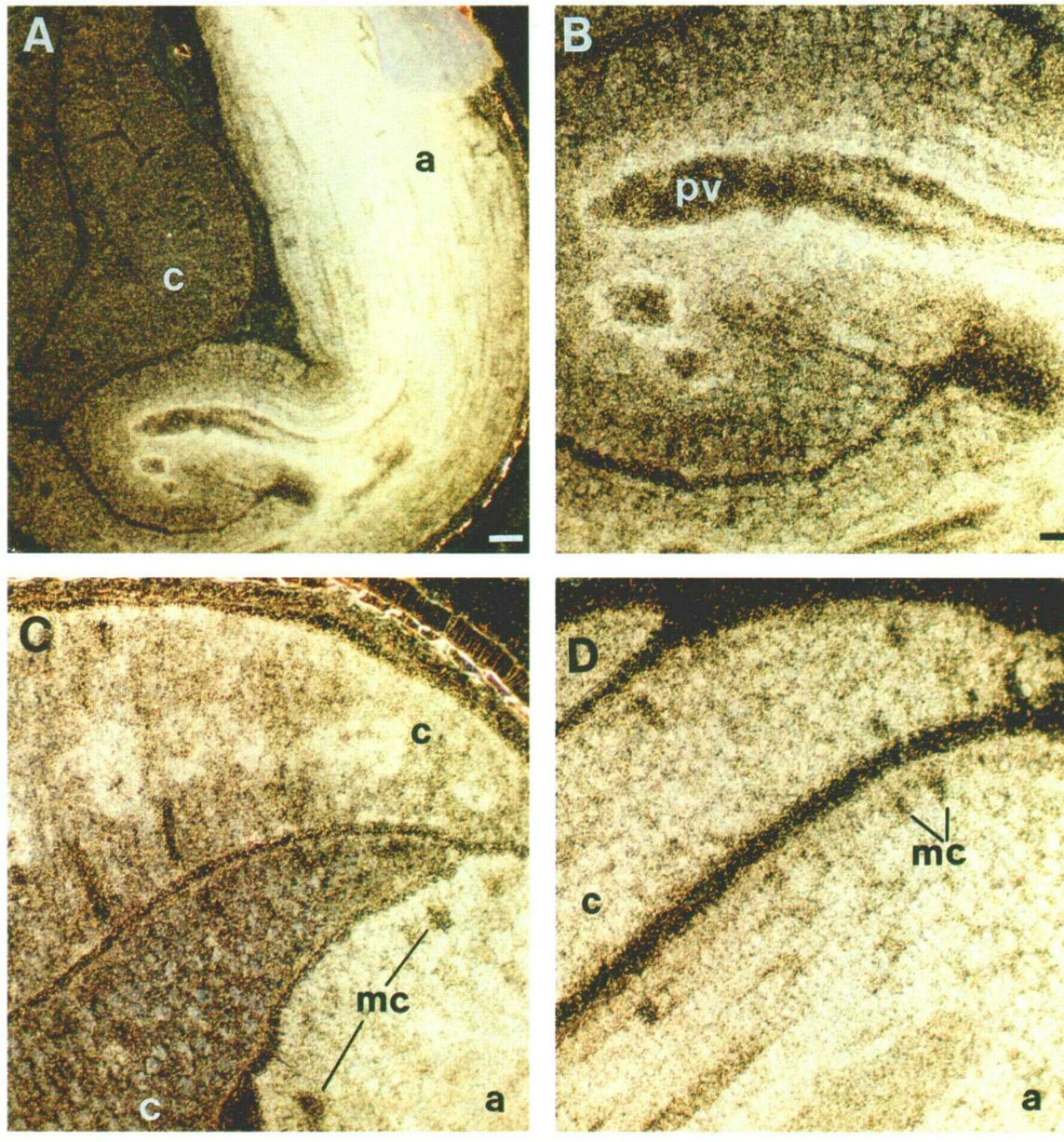

Figure 6. Dark-field images of in situ hybridization with an antisense β-glucosidase probe to sections of developing seeds of *B. napus*. A, Seeds at 35 DAP. Note the expression in the axis; no expression can be seen in the cotyledons. B, Enlargement of A showing the expression around the provascular tissue. C, Seeds at 40 DAP. Note the expression in the axis and in the outer cotyledon, especially around the provascular tissue. No expression is seen in the inner cotyledon. D, Seeds at 45 DAP. Expression is in the whole embryo, except for the myrosin cells. a, Axis; c, cotyledons; pv, provascular tissue; mc, myrosin cells. Scale bar = 125 μm in A; × 33. Scale bar = 50 μm in B, C, and D; ×84.

The deduced amino acid sequence of *B. napus* β-glucosidase was compared to those present in the SwissProt protein sequence data base using the BLAST sequence analysis program (Altschul et al., 1990). Three of the most similar sequences were selected for alignment to the *B. napus* β-glucosidase using the program PILEUP (Genetics Computer Group, University of Wisconsin, Madison) (Fig. 3). A high similarity was found between *B. napus* β-glucosidase and other plant β-glucosidases (Fig. 3). The highest identity (44%) was found with a β-glucosidase from maize (Brzobohaty et al., 1993). The identity to the three types of myrosinases in *B. napus* was 35 to 40% (Falk et al., 1992, 1995a; Thangstad et al., 1993).

Southern and Northern Hybridization

A radioactively labeled fragment corresponding to bp 975 to 1206 of the β-glucosidase cDNA was used as a probe in Southern and northern hybridizations. The probe was checked for reactivity to myrosinase cDNA clones immobilized on a filter. No cross-hybridization could be detected at the conditions used for hybridization and washing. The

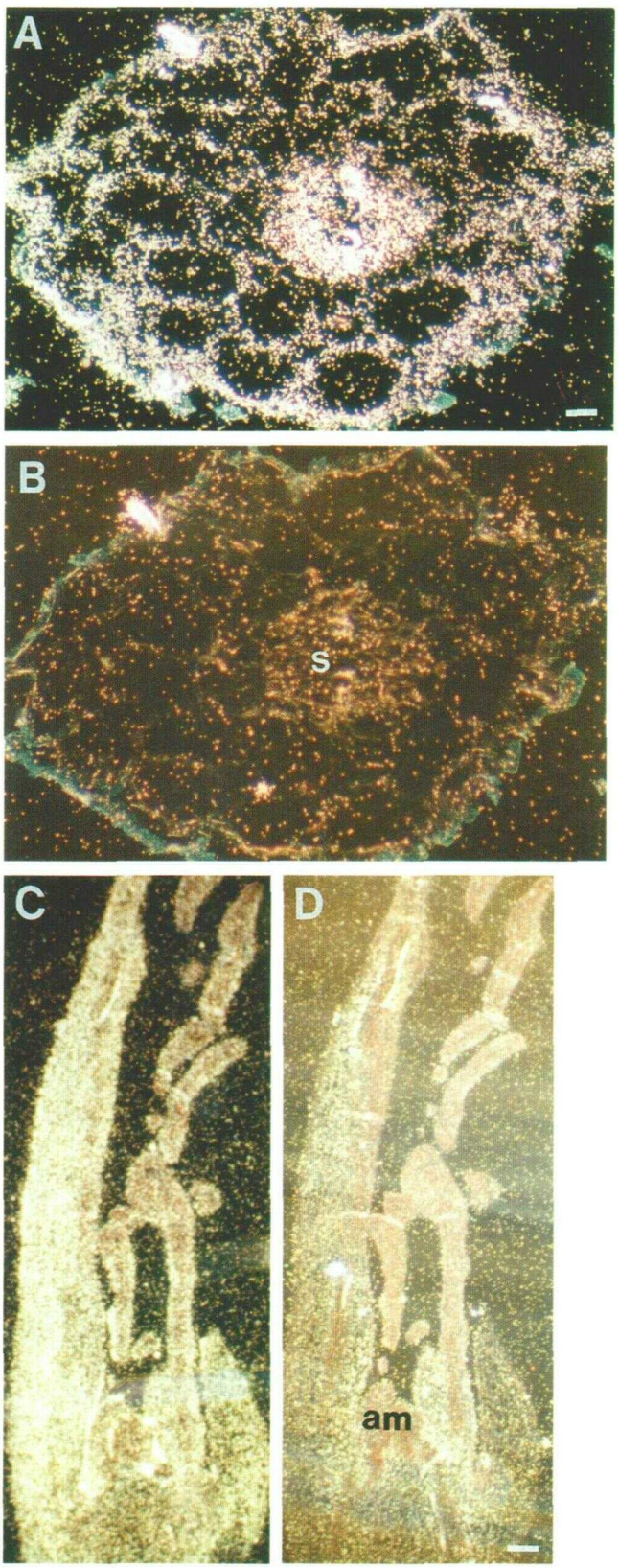

Figure 7. Dark-field images of in situ hybridizations with β-glucosidase probes to sections of tissues of *B. napus*. A, Transverse section of a root hybridized with an antisense probe. Note expression in the stele and the outer part of the cortical and epidermal cells. B, Transverse section of a root hybridized with a sense probe. C, Shoot tip hybridized with an antisense probe. Note expression in the apical meristem and the leaf primordia. D, Shoot tip hybridized with a sense probe. s, Stele; cx, cortex; am, apical meristem. Scale bar = 25 μm in A and B; ×151. Scale bar = 125 μm in C and D; ×30.

Southern hybridization of *B. napus* genomic DNA digested with three restriction enzymes showed that β-glucosidase is encoded by a small gene family, since two to five bands were encountered in the different lanes (Fig. 4). A weak but significant cross-hybridization to *A. thaliana* genomic DNA was seen (Fig. 4). Northern hybridization showed that the β-glucosidase mRNA consisted of approximately 1900 nucleotides (Fig. 5), a size compatible with the combined insert sizes of Bgl4:1 and Bgl2:1. β-Glucosidase genes were mostly expressed in the seed, with only a low amount of expression in other tissues (data not shown). The expression in the seed started at 30 DAP, with the peak at about 40 DAP (Fig. 5). This is later than the myrosinase and the cruciferin genes, which have their highest expression at 30 to 35 DAP (Falk et al., 1992; Sjödahl et al., 1993). A considerable amount of β-glucosidase mRNA was also noted at 45 DAP, at which time little myrosinase expression can be detected (Falk et al., 1992).

In Situ Hybridization

Expression of β-glucosidase genes using in situ hybridization could be detected in the embryo axis at 35 DAP (Fig. 6A). At 40 DAP, mRNA had also started to accumulate around the provascular tissue in the cotyledons (Fig. 6C). In both the embryo axis and the cotyledons, expression started around the provascular strands (Fig. 6, B and C). At 45 DAP, expression was detected in most cells of the seed (Fig. 6D). No expression could be detected in the myrosin cells at any developmental stage (Fig. 6, C and D). In situ hybridizations to tissues of developing *B. napus* plants detected β-glucosidase transcripts in young shoots, roots, and hypocotyls. In the roots, transcripts were detected in the stele, the cortex, and the epidermis (Fig. 7A), where the transcripts were preferentially detected in the periphery of the cells, which is not surprising because the cytosol in these cells is restricted to this area, whereas most of the cell volume is occupied by the vacuole. In the shoots, transcripts were detected both in the apical meristem and in the leaf primordia (Fig. 7C). Expression was also seen in the basal part of the hypocotyl, where transcripts were preferentially detected in the periphery of the cells as well (data not shown). No expression was detected in the flowers or in the seedling cotyledons. Hybridizations with sense probes to seedlings were negative (Fig. 7, B and D).

DISCUSSION

A β-glucosidase enzyme corresponding to 1 to 2% of the total β-glucosidase activity was purified from *B. napus* seeds. The enzyme(s) responsible for the major activity did not bind to a lentil-lectin column, indicating that it has a different glycosylation than the purified β-glucosidase. Two alternatives exist: either the major β-glucosidase(s) is

a differentially glycosylated form of the purified enzyme or it (they) is a separate enzyme, differing both in primary structure and glycosylation. However, a major portion of the activity in the extracts is probably accounted for by myrosinases. The purified β-glucosidase was tested for its ability to hydrolyze the naturally occurring β-glucosides zeatin-*O*-glucoside, kaempferol-3-*O*-glucoside, and cellobiose, as well as the β-thioglucoside sinigrin. Zeatin-*O*-glucoside was the only natural substrate for which the purified β-glucosidase displayed a significant activity.

An interesting finding is that although *B. napus* β-glucosidase is homologous to the myrosinases in the same species (Fig. 3), the expression of the corresponding genes is completely different. Whereas myrosinase is expressed only in the myrosin cells (Lenman et al., 1993), β-glucosidase mRNA is present in most cells in the tissues where β-glucosidase gene expression occurs. Further, the myrosin cells are distributed throughout the plant, but β-glucosidase seems to have a local expression. In the seedling cotyledons, for instance, no β-glucosidase mRNA can be detected, although a high myrosinase expression occurs in this tissue. The differences are perhaps most evident in the case of the myrosin cells in the seed, which contain a considerable amount of myrosinase but no apparent β-glucosidase mRNA, although the cells surrounding the myrosin cells show a high expression of β-glucosidase (Fig. 6). This finding further emphasizes the special nature of the myrosin cells, which do not synthesize the seed storage protein cruciferin either (Sjödahl et al., 1993).

β-Glucosidases have been implicated in plant defense through the release of toxic aglycones from glucosides, especially in the case of the cyanogenic β-glucosidases. Although the expression of these enzymes does not seem to have been studied by in situ hybridization, their distribution has been studied by immunocytochemistry. These β-glucosidases occur in mature plant tissues (Mkpong et al., 1990), and their expression does not seem to be as limited to young tissues as that of the β-glucosidase reported here. In *B. napus*, the myrosinases thus appear to be more like the cyanogenic β-glucosidases, both in tissue distribution and in possible involvement in defense reactions, than the β-glucosidase in the same species.

A β-glucosidase was recently described from maize, and its tissue distribution was studied by immunocytochemistry and northern analysis (Brzobohaty et al., 1993). The distribution of this enzyme in maize roots coincides well with the expression noted in roots for the *B. napus* β-glucosidase (Fig. 7). Of the amino acid sequences present in the EMBL data base, the maize β-glucosidase shows the highest identity (44%) to the *B. napus* β-glucosidase. The maize β-glucosidase hydrolyzed zeatin-*O*-glucoside and therefore was proposed to be involved in hormonal control of cell division in this plant. However, the specific activity of the enzyme for zeatin-*O*-glucoside was not reported. Since the primary physiological substrates of this enzyme are the hydroxamic acid glucosides (Cuevas et al., 1992; Babcock and Esen, 1994), its involvement in plant hormone metabolism cannot be the only function of the enzyme. The maize β-glucosidase is localized to the stroma of the plastid (Esen and Stetler, 1993), a localization one would not expect from a β-glucosidase involved in plant hormonal regulation. The most obvious function of the maize β-glucosidase is therefore in the release of the toxic hydroxamic acids as a response to plant injuries by insects or fungi (Babcock and Esen, 1994), in a way analogous to the myrosinases and the cyanogenic β-glucosidases.

B. napus β-glucosidase is expressed preferentially in young tissues and especially in young tissues undergoing intense cell division, as in the developing seed, the shoot tip, the basal part of the hypocotyl, and the root. This expression pattern is what would be expected from an enzyme involved in the regulation of growth and development, possibly participating in the regulation of cell division. The activity against zeatin-*O*-glucoside, which is the only natural substrate known at present for the enzyme, suggests that the enzyme serves to provide young tissues with active cytokinin from cytokinins inactivated by glucosylation. Since the mature seed is a rich source of the protein, β-glucosidase may be a key regulatory enzyme in the control of seed maturation and germination as well as in seedling development.

ACKNOWLEDGMENTS

Ms. Ulla Pihlgren is gratefully acknowledged for expert technical assistance and Dr. Stina Höglund for providing an embedded *B. napus* shoot tip. Dr. Bo Ek kindly carried out the amino acid sequence determination. Dr. Lennart Lundgren kindly provided kaempferol-3-*O*-glucoside.

Received January 12, 1995; accepted April 28, 1995.

The EMBL accession number for the sequence reported in this article is X82577.

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