

A Wound- and Methyl Jasmonate-Inducible Transcript Coding for a Myrosinase-Associated Protein with Similarities to an Early Nodulin¹

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Myrosinase is regarded as a defense-related enzyme in the Brassicaceae and is capable of hydrolyzing glucosinolates into various compounds, some of which are toxic. Several myrosinase isoenzymes exist, and some of them have been found in association with nonmyrosinase proteins. One of these associated proteins, myrosinase-associated protein (MyAP), was purified from seeds of *Brassica napus* both in complexes with myrosinase and in a free form. MyAP is a glycosylated, 40-kD protein with at least one intramolecular disulfide bridge. A monoclonal anti-MyAP antibody precipitated myrosinase activity from *B. napus* seed extracts and in these complexes both a 65- and a 70-kD myrosinase were present. The subsequent cloning and analysis revealed the existence of a gene family encoding MyAP or MyAP-related protein and that transcripts corresponding to MyAP in nonwounded plants are found predominantly in seeds. At least some members of the gene family exhibited responsiveness toward wounding and methyl jasmonate vapor. MyAP displayed considerable similarity to an early nodulin (ENOD8) from *Medicago sativa* and to a proline-rich protein (APG), described as anther specific, from *Arabidopsis thaliana* and *B. napus*. Similarity to expressed sequence tags from both *A. thaliana* and *Oryza sativa* has also been found.

The myrosinase-glucosinolate system is a preformed, two-component system that is activated upon tissue damage, whereby enzymatic decomposition of glucosinolates by the myrosinase enzyme takes place. Glucosinolates and their hydrolysis products are claimed to function as a defense against generalized herbivores and are implicated as a factor in host-plant recognition by specialized consumers (Chew, 1988; Louda and Mole, 1991). Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) consists of a group of isoenzymes capable of hydrolyzing the thioglucoside bond in glucosinolates. The resulting aglycones rearrange spontaneously and yield, in addition to sulfate, compounds such as isothiocyanates, organic thiocyanates, epithionitriles, and nitriles. Within individual plants of a species, several different types of glucosinolates exist. The exact outcome of the reaction depends on a number of factors

such as type of glucosinolate, pH, and the presence of Fe²⁺ and additional proteins (McGregor et al., 1983; Chew, 1988; Louda and Mole, 1991). The total hydrolysis profile probably also depends on the myrosinase isoenzymes present. Epithiospecifier protein (Tookey, 1973) is a protein that directs the outcome of the reaction toward epithionitrile formation when the substrate contains a terminal double bond in the side chain. Other factors, possibly proteins, that participate in the diversion of end products, e.g. nitrile and thiocyanate production, remain to be identified (McGregor et al., 1983). Glucosinolates and/or myrosinase have been found in 15 dicotyledonous families (Rodman, 1991), mainly the Brassicaceae and Capparaceae.

Myrosinases can be found in seeds, seedlings, and mature tissues, although the amount of activity and the type of isoenzymes expressed differ with respect to both the type of organ and the developmental stage (Bones, 1990; Höglund et al., 1991; James and Rossiter, 1991; Lenman et al., 1993). Within tissues, the myrosinases can be located in a few scattered, special cells, myrosin cells (Thangstad et al., 1990; Höglund et al., 1991), and within these cells the enzyme is stored inside myrosin grains (Thangstad et al., 1991; Höglund et al., 1992). In mature *Brassica napus* seeds, at least three different myrosinase isoenzymes exist (Lenman et al., 1993; Falk et al., 1995a), a 75-kD protein that occurs as a free dimer and two different myrosinases of 65 and 70 kD that are associated with other, nonmyrosinase proteins in large complexes of 200 to 800 kD (Lenman et al., 1990). Previously, two MBPs with molecular sizes of 50 and 52 kD have been characterized (Falk et al., 1995b) in seeds of *B. napus*. Furthermore, several larger but related proteins, MBPRPs, were identified by immunoreactivity and peptide sequencing. So far, no activity has been ascribed to the MBPs. It is, however, possible that one of the complexed proteins represents an epithiospecifier protein.

This preformed myrosinase-glucosinolate system is not only activated by tissue disruption but its composition is also influenced by wounding. The glucosinolate profile is changed in response to both wounding (Koritsas et al., 1991; Bodnaryk, 1992) and jasmonates (Bodnaryk, 1994; Doughty et al., 1995). Jasmonates such as MeJA have been

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

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Abbreviations: CNBr, cyanogen bromide; dap, days after pollination; MBP, myrosinase-binding protein; MBPRP, MBP-related protein; MeJA, methyl jasmonate; MyAP, myrosinase-associated protein.

implicated as endogenous plant signaling molecules that are involved in the response to stresses, such as wounding and pathogens. Jasmonates also influence many physiological processes in the plant; these aspects have recently been reviewed (Parthier, 1991; Koda, 1992; Sembdner and Parthier, 1993; Farmer, 1994; Reinbothe et al., 1994); see also Creelman and Mullet (1995).

Here we describe another protein exhibiting affinity for myrosinase complexes. This protein is derived from a gene family with wound- and MeJA-inducible members. The protein is, as shown by sequence determination and immunoreactivity, unrelated to the previously characterized MBPs and MBPRPs (Falk et al., 1995b). However, it does exhibit similarity to an early nodulin from *Medicago sativa* and to anther-specific Pro-rich proteins from *B. napus* and *Arabidopsis thaliana*. To distinguish this protein from the MBP family of proteins, we have termed it MyAP. This, however, does not necessarily imply a difference in the nature of the association.

MATERIALS AND METHODS

Plant Material and Induction of MyAP mRNA

Seeds of the *Brassica napus* var Hanna and the dihaploid line 20516 K of Svalöfs Karat were obtained from Svalöf Weibull AB (Svalöv, Sweden). Cv 20516 K was grown in a greenhouse for production of immature seeds and old stem. Old leaves were obtained from greenhouse-grown flowering plants, cv Hanna. Other tissues were harvested from cv Hanna grown in small glass jars for 3 weeks on Murashige-Skoog medium (Dixon, 1985) using a chamber maintained at 24°C and a 16-h light/8-h dark regime. For wounding studies we used leaves from either 3-week-old plants or flowering plants. Each leaf received a mechanical wound across the terminal part using a pair of grooved steel tweezers. Alternatively, 3-week-old plants were exposed continuously to MeJA (Bedoukian Research Inc., Danbury, CT) vapor. MeJA was diluted 1000 times in ethanol and 50 μ L of this solution were applied to a piece of cotton and placed in a sealed 1-L glass jar with the plant. Tissues were harvested at times indicated, frozen in liquid nitrogen, and stored at -80°C.

Purification of MyAP by Anti-MBP or by Anti-MyAP Immunoaffinity Chromatography

The anti-MBP antibody 34:14 (Lenman et al., 1990) and the anti-MyAP antibody B1H5 (see below) were purified separately from cell supernatants by affinity chromatography on protein A coupled to 1.5 g of CNBr-activated Sepharose CL-4B (Pharmacia Biosystems, Uppsala, Sweden) according to the manufacturer's instructions and poured into small columns. *B. napus* seeds (240 g) were crushed with a Waring Blendor. Twelve hundred milliliters of 20 mM imidazole-HCl, pH 6.0, 50 mM NaCl were added, and the mixture was stirred for 1 h at 4°C. The extract was centrifuged at 16,000g for 30 min and passed through a 10- μ m nylon mesh and then through a 0.45- μ m filter. The extract was then applied to the immunoaffinity column with one recycle. The bound proteins were eluted with 0.1 M Gly-

HCl, pH 3.0, 0.5 M NaCl, and the fractions were neutralized with 1 M Tris-HCl, pH 8.0. Protein-containing fractions were reduced in SDS-PAGE sample buffer containing β -mercaptoethanol as the reducing agent and alkylated with 4-vinylpyridine essentially as described by Tempst et al. (1990). The proteins were then applied to a 10% preparative SDS-PAGE gel, and after completion of the electrophoresis, MyAP could be eluted from the gel as described by Feick and Shiozawa (1990).

Purification of MyAP from a Protein-Body Fraction and Production of Monoclonal Anti-MyAP Antibodies

Protein bodies were isolated from seeds of *B. napus* using a modified version of the glycerol-based method of Begbie (1979). Four 150-g aliquots of *B. napus* seeds were crushed in 4 \times 500 mL of glycerol with a Waring Blendor. The homogenate was squeezed through a 52- μ m nylon cloth, resulting in a total volume of 1200 mL. KI (100 g) was dissolved in 290 mL glycerol to give a density of 1.45 g/mL. An 8-mL cushion of KI in glycerol was poured into 40-mL centrifugation tubes. The homogenate was layered over the cushion and centrifuged at 60,000g for 2 h at 20°C in an SRP28SA rotor (Hitachi, Tokyo, Japan). The glycerol homogenate was discarded by pipetting, leaving a 1-cm layer of homogenate over the protein-body band lying on the surface of the cushion. The tubes were refilled with homogenate and the procedure was repeated until all of the glycerol homogenate had been centrifuged. The protein-body band was transferred to two centrifuge tubes and 100% ethanol was added to fill the tubes. The protein bodies and the residual glycerol were suspended in the ethanol, and the protein bodies were pelleted at 40,000g. The protein bodies were then washed in 3 \times 60 mL of 100% ethanol. After each wash, they were pelleted at 10,000g in an SS-34 rotor (DuPont, Wilmington, DE). Protein bodies purified from 600 g of *B. napus* seeds were lysed in 30 mL of 10 mM Tris-HCl, pH 8.0, 50 mM NaCl and further extracted with 3 \times 30 mL of the same buffer.

The extract was dialyzed overnight against the same buffer. The extract was applied to a 10-mL lentil-lectin column and 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 5/5 column, equilibrated with 10 mM Tris-HCl, pH 8.0, in a fast protein liquid chromatograph (Pharmacia Biosystems). The proteins bound to this column were eluted with a 0 to 1 M NaCl linear gradient in the equilibration buffer and fractions of 1 mL were collected. For production of monoclonal anti-MyAP antibodies, 200 μ g of MyAP purified from protein bodies were emulsified in Freund's complete adjuvant and injected into the feet of four mice. Production of hybridomas was as described by Holmdahl et al. (1985). Hybridomas producing MyAP-specific antibodies were identified by the ability of the supernatants to detect purified MyAP in ELISAs. The hybridomas were subsequently grown in vitro.

PAGE and Western Blotting

Ten percent SDS-PAGE gels were prepared and run as described by Dobberstein et al. (1979). The protein samples

were reduced with 25 mM DTT, heat denatured at 95°C for 5 min, and alkylated by adding 5 μ L of 0.5 M iodoacetamide. Proteins were detected by staining with colloidal Coomassie brilliant blue G-250. Western blotting was carried out as described by Gallagher et al. (1992) and Towbin et al. (1979). The monoclonal antibodies 34:14, reacting with MBP and MBPRP, and 3D7, recognizing myrosinase isoenzymes (Lenman et al., 1990), were used.

Protein Cleavage, Isolation of Peptides, and Peptide Sequence Determination

Forty micrograms of MyAP isolated from the lysed protein bodies and 20 μ g of MyAP purified with the anti-MBP immunoaffinity column were separately digested with endoproteinase Lys C as described by Falk et al. (1995a). The resulting peptides were separated on a C₄ reversed-phase HPLC column (Applied Biosystems) using a gradient of 5 to 60% acetonitrile in 0.1% TFA. Peptides were sequenced using Applied Biosystems 470A and 476A sequencers and the PROFTA program (Applied Biosystems). One peptide, pep5, was obtained from MyAP purified using anti-MyAP immunoaffinity chromatography after CNBr cleavage (Allen, 1989).

Immunoprecipitation and Determination of Myrosinase Activity

One milliliter of protein A-Sepharose (Pharmacia Biosystems) was added per 50 mL of supernatant from hybridomas and mixed overnight at 4°C. The Sepharose was washed once with 10 mM Tris-HCl, pH 7.5, 400 mM NaCl, 2 mM EDTA, 1% Triton X-100 and three times with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl. A protein extract from *B. napus* seeds was prepared as described above, passed through a 0.22- μ m filter, dialyzed against 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and added to an excess of antibody-protein A-Sepharose and mixed for 1 h at room temperature. The gel was then washed as described above and resuspended in 1 mL of a sinigrin solution (5 mg/mL in 50 mM sodium acetate, pH 4.5). The mixture was incubated for 1 h at 37°C under gentle rotation and the Glc released from sinigrin was determined using the Glc-oxidase method Glox (Merck, Darmstadt, Germany). B1H5, 34:14, and R11.9.4 (against human β_2 -microglobulin) were used separately for immunoprecipitation and the precipitated activity was compared to the activity in a small amount of dialyzed extract. All measurements were carried out within the linear range of absorbance.

Cloning and Sequence Analysis

Degenerate oligonucleotides, based on peptide sequences, with nine extra nucleotides in the 5' end, AGTG-GATCCCAT(A/C/G/T)GG(C/T)TG(A/G/T)ATIAC(A/G)TTIGG(A/G)TT and AGTGGATCCGG(A/C/G/T)CCIATG(C/T)TIAA(C/T)GA(C/T)(C/T)T, were used for production of a PCR fragment coding for MyAP. The conditions were as follows: standard buffer as supplied by Boehringer Mannheim but supplemented with MgCl₂ up to 3 mM; 3 μ M of each primer; and as template, 40 ng of a plasmid cDNA library (Ellerström et al., 1992) made from immature

rapeseed mRNA, all in 50 μ L. The reaction was performed once at 95°C for 30 s, twice at 94°C for 15 s, 46°C for 25 s, and 1.5 min ramp time to 72°C, then at 72°C for 1.5 min, 35 times at 94°C for 15 s, 55°C for 15 s, 72°C for 1.5 min, on a GeneAmp 9600 (Perkin-Elmer Cetus, Norwalk, CT). The resulting PCR product, which corresponded to amino acid residues 173 to 274 (see "Sequence Analysis of cDNA Clones Corresponding to MyAP"), was labeled with [γ -³²P]CTP by random priming (Gelfand et al., 1992) before being used as a probe to screen (Sambrook et al., 1989) the cDNA library from which it had been derived. Inserts of the clones were recloned into pBluescript II SK+ (Stratagene) and plasmids were prepared using alkaline lysis and LiCl precipitation (Sambrook et al., 1989) before both strands were sequenced using a *Taq* DyeDeoxy terminator cycle sequencing protocol essentially as described by Applied Biosystems. Sequence reactions were run on an Applied Biosystems 373A DNA sequencer. All sequences were managed and analyzed using the Staden package (R. Staden, MRC Laboratory of Molecular Biology, Cambridge, UK, e-mail rs@mrc-lmb.cam.ac.uk). Sequences were aligned using the program Clustal V (Higgins et al., 1992) and a PAM250 matrix. Identical and similar amino acids in the sequence alignment figure were shaded using the program Boxshade (K. Hofmann, Bioinformatics Group, Lausanne, Switzerland, e-mail khofmann@isrec-sun1.unil.ch).

Northern and Southern Hybridizations

Total RNA was extracted using the phenol-SDS method as previously described by Falk et al. (1992) and 10 μ g per sample were denatured and subjected to electrophoresis through a formaldehyde-containing 1.4% agarose gel (Sambrook et al., 1989). RNA was transferred to Hybond C-extra membrane (Amersham) according to the manufacturer's instructions. The Southern filter with genomic DNA was as described by Rödin et al. (1993). Both Southern and northern filters were hybridized (Sambrook et al., 1989) in 6 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 \times Denhardt's solution, 0.1% SDS, 100 μ g/mL single-stranded salmon sperm, with the PCR fragment described above as a probe (see "Cloning and Sequence Analysis") and washed in 2 \times SSC, 0.1% SDS at room temperature followed by 1 \times SSC, 0.1% SDS, at 65°C for 30 to 60 min (northern blot for seed time curve and Southern blot) or 2 \times SSC, 0.1% SDS at 65°C for 30 min (northern blot for tissue distribution and induction studies). Additionally, the northern filter for tissue distribution and induction studies was subjected to a high-stringency wash (data not shown); this corresponds to 0.2 \times SSC, 0.1% SDS at 65°C for 30 min. Equal loading was tested by hybridizing the filters to an rRNA probe derived from *Vicia faba* (Yakura et al., 1984). Signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Purification and Characterization of MyAP

The 70- and 65-kD myrosinases have previously been found to form large complexes with MBPs (Lenman et al., 1990), and later a 40-kD protein, MyAP, was found to be

associated with these complexes (Falk et al., 1995a). In contrast, MBPRPs were barely detectable in complexes immunoprecipitated using a polyclonal antiserum against myrosinase (Falk et al., 1995b). To further characterize the complexes formed between the 70- and 65-kD myrosinases and other proteins, myrosinase complexes were isolated. Two hundred forty grams of seeds were used to obtain approximately 100 μ g of MyAP from the myrosinase complexes purified by immunosorbence using the anti-MBP antibody 34:14, as judged from the staining of gels with Coomassie brilliant blue. To separate MyAP from the complexes we had to use SDS, however, which meant that the proteins were denatured. Also, the conditions used for the elution of the proteins from the immunoaffinity column possibly denatured the proteins. We therefore wanted to purify MyAP under milder conditions. From previous attempts to purify myrosinase grains (A. Falk, unpublished data), we had reason to believe that MyAP could be purified from a protein-body fraction, which could be enriched using the glycerol-based method of Begbie (1979). This isolation protocol yielded a total of 260 mg of protein-body proteins from 600 g of *B. napus* seeds, and from these 260 mg of protein, 3.1 mg of glycoproteins were isolated by lentil-lectin chromatography. The glycoproteins were further purified by ion-exchange chromatography on a Mono Q 5/5 column, pH 8.0, as shown in Figure 1. MyAP eluted as a broad peak at 0.2 to 0.6 M NaCl, and a total of 0.7 mg MyAP was obtained. It should be noted that the absorbance peak at an effluent volume of 13 mL did not correspond to MyAP but to an unidentified compound with a maximal absorption at 250 nm, possibly bound to a con-

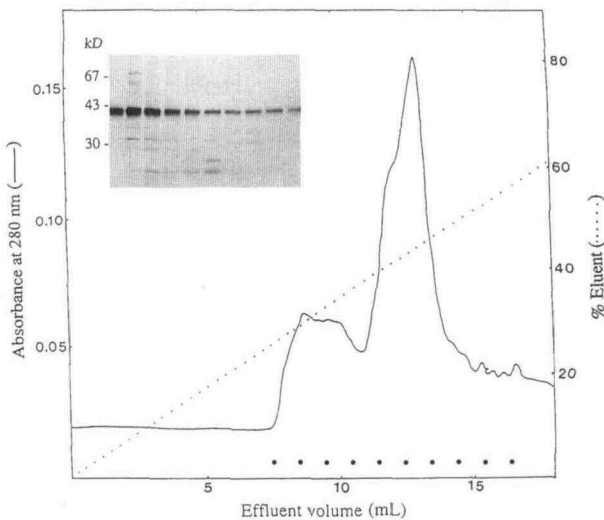


Figure 1. Ion-exchange chromatography on a Mono Q 5/5 fast protein liquid chromatography column at pH 8.0. Proteins were extracted from a protein-body fraction, and MyAP was enriched by lentil-lectin affinity chromatography. The eluate from the lentil-lectin column was applied to the Mono Q column and eluted with a 0 to 1 M linear NaCl gradient. A Coomassie brilliant blue-stained SDS-PAGE gel of fractions from the ion-exchange chromatography is shown in the inset and dots under the elution profile correspond to successive lanes in the gel.

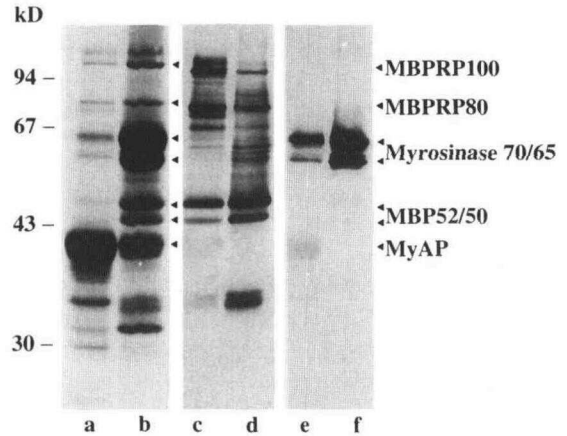


Figure 2. SDS-PAGE gel of reduced and alkylated proteins eluted from an anti-MyAP immunosorbent column (lanes a, c, and e) or an anti-MBP immunosorbent column (lanes b, d, and f). Proteins were stained with Coomassie brilliant blue (lanes a and b) or immunoblotted with 34:14 (lanes c and d) or 3D7 (lanes e and f) antibodies.

taminating glycoprotein present in low amount in the corresponding fractions.

MyAP isolated from protein bodies as well as that purified by immunosorbent chromatography were separately digested with Lys C, and the resulting peptides were purified by reversed-phase chromatography. Four peptides (pep1 to pep4) were sequenced, and of these two were shown to be identical in MyAP isolated from the two sources. The peptide sequences and the highly similar elution profiles of the generated peptides indicated that the two proteins were identical, and it was therefore reasonable to designate both of them MyAP. One peptide (pep5) was obtained after CNBr cleavage of material purified by anti-MyAP immunoaffinity chromatography. MyAP contained at least one internal disulfide bridge, because the mobility of the protein in SDS-PAGE gels decreased after reduction with DTT (Falk et al., 1995a). The protein was evidently glycosylated because the mobility of the protein in SDS-PAGE gels increased as a result of deglycosylation with peptide-N-glycosidase F and endoglycosidase H (Falk et al., 1995a). No mobility shift could be detected with O-glycosidase.

Immunoprecipitation of Myrosinase Activity and Immunoaffinity Purification of Myrosinase Complexes

To further characterize MyAP, the protein was injected into mice to produce monoclonal antibodies. Four anti-MyAP antibodies were identified as reacting positively with purified MyAP in ELISA. However, no antibody could be found that reacted positively with MyAP by western blot analysis. The ability to precipitate myrosinase activity was tested for one of these antibodies (B1H5). This antibody precipitated 1.5% of the myrosinase activity present in the extract; the corresponding value for 34:14 was 38%. The activity precipitated by B1H5 was increased to only 1.8% by omitting the high-stringency wash.

Myrosinase complexes were isolated using either the anti-MBP antibody 34:14 or the anti-MyAP antibody B1H5, separated by SDS-PAGE, and analyzed by western blotting using

two different antibodies (Fig. 2). Both 34:14 and B1H5 were capable of affinity purifying myrosinase isoenzymes with molecular masses of approximately 65 and 70 kD and, in addition, two minor isoforms with molecular masses in between, as judged by the reaction with the myrosinase-specific 3D7 antibody (Fig. 2, lanes e and f). As expected, the protein fraction isolated on the anti-MBP immunosorbent column contained MBP50, MBP52, and several MBPRPs (Fig. 2, lane d). It is interesting that the fraction recovered from the anti-MyAP column also contained MBPRPs (Fig. 2, lane c). This is in contrast to myrosinase complexes purified by immunosorbent chromatography using anti-myrosinase antibodies in which these proteins were hardly detectable by western blotting with the 34:14 antibody (Falk et al., 1995b). The relative amount of MyAP to myrosinases present in protein fractions isolated by immunosorbent chromatography on anti-MyAP and anti-MBP columns (Fig. 2, lanes a and b) suggested that MyAP is primarily present in a myrosinase-free form. Coomassie staining of lanes run in parallel showed that the two isolated fractions contained some low-molecular-weight proteins that did not react with either 34:14 or 3D7 (Fig. 2, lanes a and b).

Sequence Analysis of cDNA Clones Corresponding to MyAP

To obtain a probe suitable for screening a *B. napus* cDNA library, several degenerate oligonucleotides, based on pep-

tide sequences, were constructed for use in PCR with the same cDNA library as the template. One of the primer pairs yielded a DNA fragment of approximately 300 bp that was subsequently used as a probe for the isolation of several clones corresponding to MyAP, and among these two of the longest ones (Fig. 3) were chosen for further characterization, MyAP5 (1237 bp) and MyAP4 (1080 bp). MyAP4 was truncated in the 5' end at an internal *Xho*I site, whereas MyAP5 seemed to encode a complete MyAP molecule including a potential signal peptide sequence (von Heijne, 1984). The 125-bp 3' untranslated region of MyAP4 contained two potential polyadenylation signals 26 bp apart. The 3' untranslated region of MyAP5 was identical with that of MyAP4 with the exception that the 5'-most polyadenylation signal seemed to have been functional in the corresponding mRNA. However, it cannot be excluded that the polyadenylation signal located closest to the 3' end and the A-rich sequence around it might have hybridized the oligo(dT), which was used to prime cDNA synthesis, during construction of the library. The two clones were also identical in the coding sequence except for the fact that MyAP4 harbored a 36-bp sequence that was missing from MyAP5. The derived amino acid sequence of MyAP5 embodied five potential *N*-glycosylation sites (one in the putative signal peptide) and six Cys residues, two of which were located in the potential signal peptide; the predicted amino acid sequence of MyAP4 contained two extra Cys residues and one additional putative *N*-glycosylation site

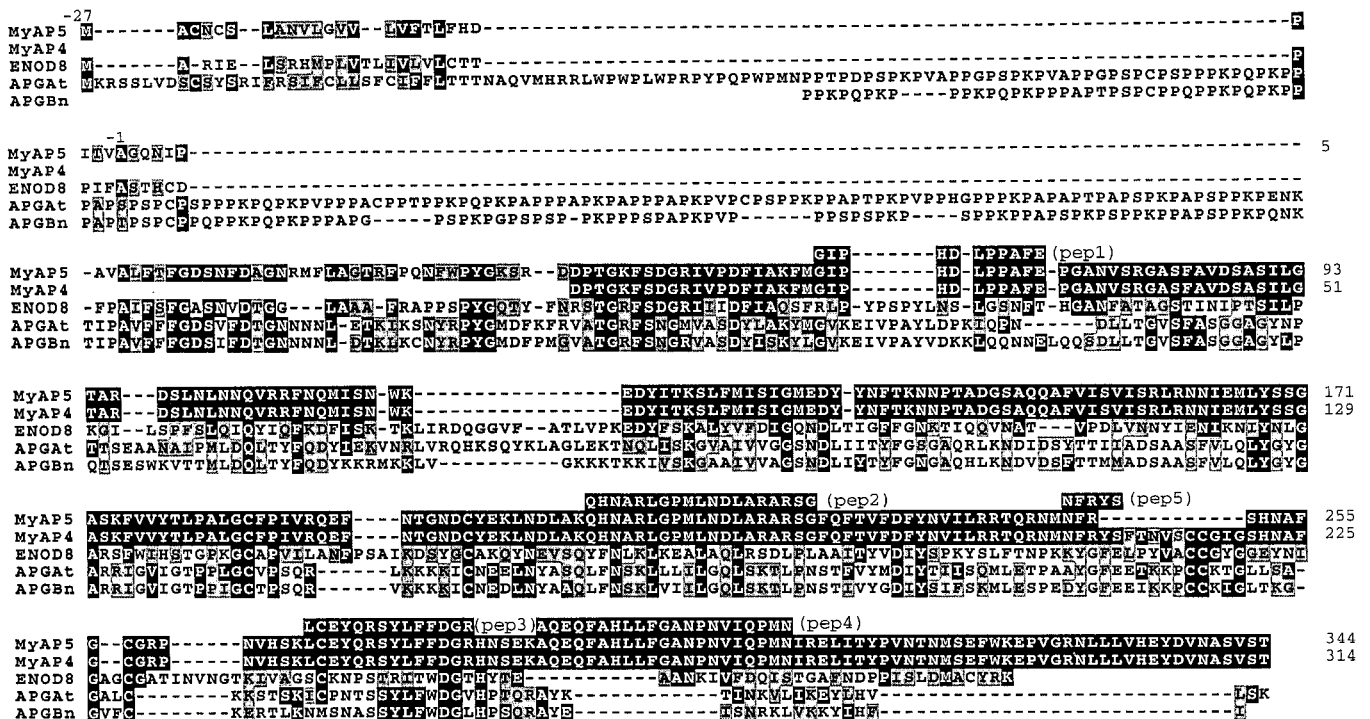


Figure 3. The MyAP amino acid sequence predicted from the nucleotide sequences of the cDNA clones MyAP4 and MyAP5 is compared to protein sequences derived from clones corresponding to an early nodulin from *M. sativa* (ENOD8) and to an anther-specific Pro-rich protein present in both *A. thaliana* and *B. napus* (APGAt and APGBn, respectively). Amino acids from -27 to -1 in the protein sequence derived from the cDNA clone MyAP5 correspond to a putative signal peptide. MyAP peptide sequences are inserted above the MyAP5 protein sequence. Horizontal bars indicate gaps introduced to optimize the alignment. Identical amino acids are shown in black boxes, whereas gray boxes indicate conserved amino acids.

as a result of the additional 36 bp. The calculated molecular weight of the protein encoded by MyAP5, excluding the putative signal peptide, was approximately 39,000. The derived amino acid sequence agreed completely with the sequences of five peptides isolated from MyAP (Fig. 3). The derived protein sequences were compared to other sequences using the BLAST server provided by the National Center for Biotechnology Information (Bethesda, MD, e-mail blast@ncbi.nlm.nih.gov).

From the data bases several sequences exhibiting similarity to MyAP were identified (Fig. 3), an early nodulin (ENOD8) from *M. sativa* (Dickstein et al., 1993) and an anther-specific Pro-rich protein (APG) present in both *A. thaliana* and *B. napus* (Roberts et al., 1993). MyAP and ENOD8 share 26% amino acid identity and, in addition, 26% amino acid similarity, and MyAP and the *A. thaliana* APG protein share 24% amino acid identity and, in addition, 25% amino acid similarity. The corresponding figures for the *B. napus* APG seemed to be somewhat lower. The similarity between MyAP and ENOD8 was less than the similarity between APG and ENOD8 (30% identity and 38% similarity). However, the similarity matrix used was not specified. A potential signal peptide was reported for both the ENOD8 and the APG protein, and a high degree of similarity existed between the signal peptide sequences of MyAP and ENOD8, with the peptide cleavage site predicted at equivalent positions. Cys residues in the MyAP sequence appear to be conserved in the other sequences, except in one instance in which no Cys residue exists at the corresponding position in *B. napus* APG. Both the proteins corresponding to APG and the protein corresponding to ENOD8 contain additional Cys residues.

Recently, a new cDNA sequence, EP4a (van Engelen et al., 1995), was deposited in the GenBank data base, and the protein sequence derived from this clone shared 26% amino acid identity and an additional 30% amino acid similarity with the MyAP. The amino acid sequence identity between the EP4a and the ENOD8 proteins was reported to be 51%. Data base searches also revealed similarities of the MyAP to the translated sequence of expressed sequence tags from both *A. thaliana* and *O. sativa*, e.g. a 70% identity to a 100-amino acid stretch from *A. thaliana* (accession No. T45993) and a 50% identity to a 55-amino acid stretch from *O. sativa* (accession No. D40539).

Difference in Sequence between MyAP4 and MyAP5 Appears to Be the Result of Differential Splicing

Given the fact that the sequences of MyAP4 and MyAP5 are identical except for the insertion/deletion, a possible explanation for their occurrence is alternative splicing of a common primary transcript. Another possibility is that the MyAP4 and MyAP5 mRNAs might be encoded by two different, although almost identical, genes. In an attempt to resolve this question, we performed PCR on *B. napus* genomic DNA using primers flanking the area containing the insertion/deletion. This resulted in the amplification of one major DNA fragment, and direct sequencing of this fragment (Fig. 4) showed that the amplified area contained one intron located in connection to the 5' side of the insertion/deletion point. The sequence surrounding the two borders of the 36-bp sequence seemed to follow the consensus for a plant 3' splice site equally well (Filipowicz et al., 1996). However, in a majority of transcripts, at least in seeds of *B. napus*, the most 5' site was used. Also, one of the peptide sequences (pep5) was derived from a MyAP harboring the corresponding amino acid sequence. The exon/intron structure in this area appears to be conserved between the *A. thaliana* APG gene (Roberts et al., 1993) and the gene corresponding to MyAP4(5).

MyAP4 and MyAP5 Are Primarily Expressed in Seed and Are Derived from a Gene Family with Wound- and MeJA-Responsive Members

MyAP4 and MyAP5 appear to belong to a gene family that, depending on the restriction enzyme used, gave rise to 3 to 5 strongly and 3 to 10 weakly hybridizing bands with *B. napus* genomic DNA using a probe encoding amino acid residues 173 to 274 of MyAP4 (Fig. 3). In *A. thaliana* genomic DNA, 3 to 4 bands were detected (Fig. 5). In this context, it is worth mentioning that ENOD8 is reported to be a multiple copy gene. Northern blot hybridizations revealed that MyAP is preferentially expressed in immature seeds with a broad maximum from 30 to 40 dap (Figs. 6 and 7). It should, however, be noted that the lane corresponding to seeds at 35 dap in Figure 7 was overloaded approximately 5 times, as compared to the other lanes. Still, the amount of transcript in seeds at 35 dap (Fig. 7, lane a) was approximately 2.5 times higher than in pericarp at 35 dap (Fig. 7, lane b). The transcript measured approximately

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ATTCTTCGCAGGACACAAAGAAACATGAATTTTCgtaagtattatgtaccgttacaaaata
197 I L R R T Q R N M N F
tatacactagttaatataaaggatttggactagatttgataatatttgtgtgactgac

tagGTTACTCCTTTACGAATGTATCGTGCTGCGGTATAGGTCGCACAACGCCCTTCGGTTG
R [Y S F T N V S C C G I G] S H N A F G C 227

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Figure 4. Genomic nucleotide and translated sequence from the area corresponding to a region surrounding the insertion/deletion point of MyAP4 and MyAP5. Exons are in uppercase letters, whereas the intron is displayed in lowercase letters. Boldface letters follow the consensus sequence for 5' and 3' splice sites in plants. The amino acids present in MyAP4 but absent in MyAP5 are enclosed by brackets. Numbers indicate the corresponding amino acids in the protein sequence translated from the cDNA clone MyAP4.

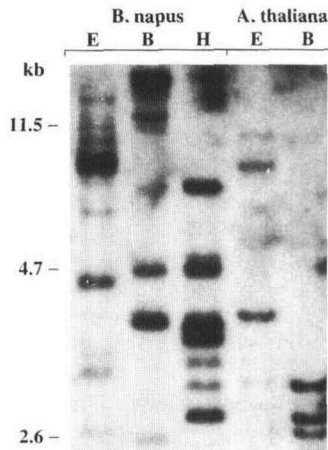


Figure 5. Southern blot analysis of MyAP genes in *B. napus* and *A. thaliana*. The enzymes used were *Eco*R I (E), *Bam*HI (B), and *Hind*III (H). A DNA size marker is indicated in kb at the left.

1400 nucleotides in size, which agrees well with the 1225-bp [excluding the poly(A) tail] insert of MyAP5. In tissues other than the seed the relative amount of MyAP transcript was found to be as follows (Fig. 7): pericarp (35 dap) > root \approx hypocotyl > primary leaf. A weak signal could be detected in stem, old leaf, and cotyledon. However, the expression exhibited a substantial responsiveness to mechanical wounding and MeJA vapor in cotyledons. The amount of transcript in cotyledons after MeJA treatment (Fig. 7, lane j) was approximately 5 times higher than the amount present in seeds from noninduced plants at 35 dap (Fig. 7, lane a). A slight increase in the amount of transcript was also observed in old leaves after wounding (Fig. 7). High-stringency washes of the northern filter resulted in the preferential loss, relative to the seed, of signals from pericarp and MeJA-exposed cotyledon, and no signal could be detected in the lanes corresponding to the other tissues (data not shown).

To investigate the presence of transcripts corresponding to the two clones MyAP4 and MyAP5, primers flanking the area containing the insertion/deletion were used to PCR amplify MyAP-specific first-strand cDNA made from *B. napus* seed mRNA. MyAP4 was identified as the major transcript in seeds from 30 to 45 dap. However, a weak band corresponding in size to MyAP5 was also detected (data not shown).

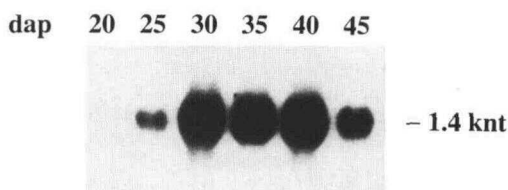


Figure 6. Northern blot analysis of total RNA from *B. napus* seeds harvested on different daps. MyAP transcripts were detected with a PCR probe and the transcript size, estimated from an RNA marker, is given in kilonucleotides (knt) on the right.

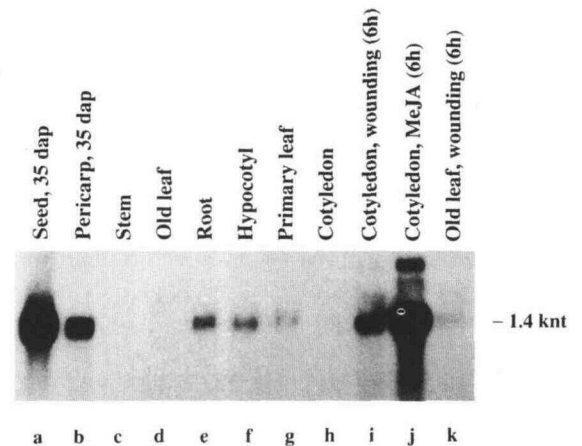


Figure 7. Northern blot analysis of total RNA from different organs of *B. napus*. MyAP transcripts were detected with a PCR probe and the transcript size, estimated from an RNA marker, is given in kilonucleotides (knt) at the right. The RNA in lanes e to j is from approximately 3-week-old plants, whereas RNA in lanes c, d, and k is from flowering plants.

DISCUSSION

To further clarify the role of proteins associated with myrosinases isolated from *B. napus* seeds, we have characterized a new protein, MyAP. Apart from myrosinases and MyAP, these complexes also contain another protein, MBP. Myrosinase-containing complexes can be isolated by immunosorbent chromatography or immunoprecipitation using monoclonal antibodies against MBP and MyAP. In complexes purified using an anti-MBP antibody, the amount of MyAP seemed to be approximately equal to the amount of MBP. In spite of this, the anti-MyAP antibody precipitated considerably less myrosinase activity than did the anti-MBP antibody. This might indicate that MyAP is less accessible to the anti-MyAP antibody than MBP is to the anti-MBP antibody. The somewhat contradictory results with the two antibodies could be reconciled by the existence of different subpopulations of myrosinase complexes. The anti-MyAP antibody, when used in immunosorbent chromatography, might enrich for complexes with more exposed MyAP and with relatively more MBP-PRP. However, these conclusions should be regarded as tentative, since antibodies might induce dissociation of protein from the myrosinase complex. It is also possible that the anti-MyAP antibody indirectly caused an inactivation of myrosinases by binding to the complexes. Comparison of the composition of the complexes isolated by immunosorbent chromatography using anti-MBP and anti-MyAP antibodies also suggested that MyAP occurs mainly in free form.

Isolation and characterization of cDNA clones showed that two major MyAP transcripts, MyAP4 and MyAP5, exist, differing only by an insertion/deletion of 36 bp that arises from alternative splicing of a common primary transcript. Peptide sequencing has so far confirmed the existence of a protein corresponding only to the larger form, MyAP4, and we do not know if the MyAP5 is stable. If both proteins exist, they should display different properties. The

insertion encodes two adjacent Cys residues that are not able to form a common disulfide bridge (Schulz and Schirmer, 1979), and consequently, they will either be free and reactive or be engaged in disulfide bonds with other Cys residues present in MyAP or in other proteins. It should be noted that MyAP is noncovalently bound in the myrosinase complexes. Thus, if the two additional residues are engaged in intramolecular disulfide bridges in the MyAP4, the lack of these residues in a protein translated from a MyAP5-like mRNA should have a definite impact on the structure of this protein.

Another factor contributing to the heterogeneity of MyAP is the indication from Southern blotting experiments that MyAP is encoded by a gene family in both *B. napus* and *A. thaliana*. Furthermore, the different signal strengths of the hybridizing bands suggest that some members of this gene family are more distantly related to the MyAP4/MyAP5 gene. Indeed, the preferential loss of hybridizing signal under high-stringency conditions in northern hybridization of RNA from vegetative tissues might be an indication that the MyAP4/MyAP5 gene is seed specific and that MyAP-like genes are expressed in other tissues or under other circumstances. Possibly some of the MyAP (or related) transcripts in the healthy plant are regulated by endogenous jasmonates. However, to our knowledge the only data concerning endogenous jasmonates in *B. napus* is from seed (Wilén et al., 1990). In addition, it is not always the case that high amounts endogenous jasmonates correspond to high levels of jasmonate-inducible transcripts (Creelman and Mullet, 1995). Also, certain responses to jasmonates seem to be possible only at specific developmental stages (Farmer, 1994).

Highly significant similarities have been found to the product of the APG gene from *A. thaliana* and *B. napus*, which is suggested to play a role in gametogenesis, to the EP4 protein, which is secreted by carrot suspension cells, and to the ENOD8 protein, an early nodulin from *M. sativa*. Early nodulins are thought to be involved in nodule organogenesis and in the nodule invasion process. Also, during the nodulation process, protein expression reminiscent of a defense response against a pathogenic attack takes place. It has, however, been argued that this is a nonspecific response that rather should be regarded as a stress reaction (McKhann and Hirsch, 1994).

Several proposals can be made for a possible function of MyAP within the myrosinase system. It may affect the stability, the activity, or the specificity of the myrosinases or function as an accessory protein specifying the outcome of the glucosinolate hydrolysis. It is currently not known whether the myrosinase complexes are deposited as such in the myrosin cells in the seed or are formed only after tissue disruption. Also, so far we have not investigated the presence of myrosinase complexes in homogenates of vegetative *B. napus* tissues. However, a wound-induced increase in MyAP-related proteins might represent an indirect way to modify the myrosinase activity, perhaps to bias the activity toward the glucosinolates that are induced by wounding (Koritsas et al., 1991; Bodnaryk, 1992). The preformed myrosinase enzymes exhibited the least activity

toward these wound-induced glucosinolates (James and Rossiter, 1991). Under the conditions used in this study, transcripts corresponding to the MB gene family of myrosinases present in vegetative tissues (Falk et al., 1992) displayed only a slight, if any, increase after wounding or MeJA treatment (data not shown). Nevertheless, even a slight increase might be of importance, especially if this increase results in the expression of new isoenzymes with different substrate specificity. It cannot be excluded that the association of MyAP with myrosinase complexes is a fortuitous event during homogenization and solubilization of seed proteins, and in that case the function of the protein should be sought outside the myrosinase-glucosinolate system. However, the wound inducibility of MyAP or related proteins indicates a role in the defense against pests or herbivores, like the function proposed for the myrosinase-glucosinolate system.

ACKNOWLEDGMENTS

Bo Ek is gratefully acknowledged for help with protein sequence determination and we thank Elfi Öhren for help with the production of monoclonal antibodies.

Received July 31, 1995; accepted November 8, 1995.

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