cDNA Cloning of Radish (Raphanus sativus) Myrosinase and Tissue-Specific Expression in Root

Masakazu Hara, Yoko Fujii, Yuri Sasada and Toru Kuboi

Department of Environmental Science for Human Life, Faculty of Agriculture, Shizuoka University, 836 Ohy, Shizuoka, 422-8529 Japan

Two cDNA clones of myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) were isolated from radish (Raphanus sativus) seedlings. Both clones were identified as MB (B type myrosinase) from their sequence homology at the amino acid level to MBs cloned from other Brassicaceae species. The tissue distribution of gene expression and enzyme activity of myrosinase corresponded well to the site of glucosinolate accumulation in different tissues of radish. The myrosinase-glucosinolate system was localized in the cotyledons in the seedlings and in the peel of the root in the mature plant. Tissue printing analysis showed that myrosinase mRNA and activity were localized in the epidermis and vascular cambium that were present in the peripheral part of the root but few signals were detected in the parenchyma inside of the vascular cambium. Since the myrosinase-glucosinolate system is known to be a defense system in higher plants, the localization of the myrosinase-glucosinolate system in the peel of the root may act to protect the sink organ from the attack of herbivores or pathogens in soil.

Key words: Glucosinolate — Myrosinase — Radish (Raphanus sativus) — Thioglucoside glucohydrolase (EC 3.2.3.1) — Tissue printing.

Abbreviations: RMB, radish myrosinase B type; MA, MB and MC, A, B and C type myrosinase; RT-PCR, reverse transcription PCR; PVDF, polyvinylidene difluoride.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AB042186 (RMB1) and AB042187 (RMB2).

Introduction

The myrosinase-glucosinolate system is considered to be a defence system in Brassicaceae species (Fenwick et al. 1983, Bennett and Wallsgrove 1994). The system consists of two components, the enzyme myrosinase (β-thioglucosidase, thioglucoside glucohydrolase, EC 3.2.3.1) and the substrates glucosinolates which are sulfur-containing glucosides that are not toxic (Fig. 1). In uninjured plants, myrosinase and glucosinolates are kept sequestered from one another. When the plant tissue is damaged mechanically, the enzyme and substrates are mixed together, producing the cleavage products, isothiocyanate, nitrile and thiocyanate, which have potent antimicrobial effects or influence insect behavior (Chew 1988a, Chew 1988b). Allyl isothiocyanate inhibited the growth of many species of bacteria and yeasts in vitro (Brabban and Edwards 1995, Kyung and Fleming 1997). Some isothiocyanates attracted crucifer specialists but were toxic to insects which ate other species (Chew 1988a). Hydrolytic products of indole glucosinolates are precursors of indoleacetic acid (Bartel and Fink 1994) or Brassicaceous phytoalexins (Monde et al. 1994).

From the end of the 19th century, myrosinase has been believed to be present in a special type of secretory cell, the myrosin cell, that is observed only in the early stage of germination (Bones and Rossiter 1996). Phelan and Vaughan (1980) reported that considerable myrosinase activity was detected in organs of Sinapis alba containing no myrosin cells and concluded that the enzyme could exist in non-myrosin cells. Kelly et al. (1998) demonstrated by the double labeling method using anti-myrosinase and anti-sinigrin antibodies to co-localize both myrosinase and glucosinolates, that myrosinase was present in both myrosin and aleurone cells but glucosinolates were localized only in aleurone cells in the cotyledons of Brassica juncea. Myrosinase and glucosinolates were considered to be localized in the cytoplasm and vacuole, respectively (Bones and Rossiter 1996, Kelly et al. 1998). Myrosinase forms complexes with myrosinase-binding protein in seeds (Takechi et al. 1999).

Recently, myrosinase genes have been cloned from oilseed rape (B. napus) (Falk et al. 1992), turnip (B. campestris) (Machlin et al. 1993), leaf mustard (B. juncea) (Heiss et al. 1999), white mustard (Sinapis alba) (Xue et al. 1992), and thale cress (Arabidopsis thaliana) (Xue et al. 1995) and classified into four subtypes, MA, MB, MC and TGG, on the basis of amino acid sequences (Rask et al. 2000). In B. napus, MB showed dominant expression in seeds, seedlings, young leaves, and other organs in the mature plant, whereas MA and MC were expressed only in developing seeds (Lenman et al. 1993, Falk et al. 1995). MA and MB clones were also isolated from S. alba and the X-ray structure of MA was elucidated (Burmeister et al. 1997). Two genes, tgg1 and tgg2, isolated from A. thaliana could not be classified into either MA, MB nor MC (Xue et al. 1995). Both genes were expressed in leaf and flower, but only tgg2 transcripts were found in developing seeds.

1 Corresponding author: E-mail, masahara@agr.shizuoka.ac.jp; Fax, +81-54-238-4881.
The root is the main accumulation site of myrosinase activity in the vegetative tissue of mature *B. napus* plants (Bones 1990). In addition, the glucosinolate content in the root was higher than that in the top of *B. campestris* (Carlson et al. 1987). Despite the importance of roots for the accumulation of myrosinase and glucosinolate, little research has been done on the tissue specific localization of myrosinase and glucosinolate in the root of Brassicaceae plants. In this paper, we report the distribution of myrosinase gene expression, enzyme activity, and glucosinolate content in the swollen root of radish (*Raphanus sativus*). We also discuss the functional feature of the myrosinase-glucosinolate system in the sink organ of radish.

**Materials and Methods**

**Plant materials**

Seeds of radish (*R. sativus* L. cv. comet) were sown on well-wetted filter paper with water in plastic trays. Five-day-old seedlings grown under continuous light were used for extraction of total RNA, crude enzyme and glucosinolate. Mature plants of radish harvested in the morning were purchased from a market and used for experiments within the same day.

To analyze the distribution of myrosinase gene expression, myrosinase activity and glucosinolate content, we cut the cotyledons, hypocotyl and root from the seedlings with a razor. Leaves and roots of mature plants were separated with a razor. Using a well-sharpened knife, peel of the root consisting of epidermis was carefully sliced. The incision was made at the outer part of cortex without wounding the epidermis. When the root was separated into three portions, the top, middle and bottom, the root was cut transversely at two sites at equal distances on the vertical length.

**cDNA cloning of radiish myrosinase**

Total RNA was extracted from whole seedlings of radiish by the phenol/SDS method (Hussey and Hunsperger 1996) and precipitated with LiCl. Poly(A)^+ RNA was isolated from the total RNA using a polyA Spin mRNA Isolation Kit (New England BioLabs). A cDNA library was constructed using the cDNA Synthesis, ZAP-cDNA Synthesis and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. cDNA fragment of radiish myrosinase for library screening was prepared by RT-PCR. The first strand cDNA was synthesized from poly(A)^+ RNA of radiish seedlings. The target region within the myrosinase cDNA was subsequently amplified using an RNA PCR Kit Ver. 2.1 (Takara). Reverse transcription was carried out at 42°C for 30 min and the PCR reaction was achieved through 35 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 90 s. Three degenerated primers, designed on the basis of three conserved regions of myrosinases from *B. napus* (MA: Z21978, MB: X60214 and MC: X59879) and *A. thaliana* (TGG1: X71914 and TGG2: X71915), were used for nested PCR. PCR with primer 1 (5’-TTRYMRAAYTCTARTTRTC-NCC-3’) and primer 3 (5’-TAYATHTGNGCNACAYAAYC-3’) gave a predicted fragment of 740 bp. The fragment was subjected to PCR again with primer 1 and primer 2 (5’-AAYTAYTAYGNNACAR-TAYGC-3’) and a 420 bp fragment was amplified. The product, cloned into the pGEM-T Easy plasmid vector (Promega), was proved to be a part of myrosinase cDNA by sequencing and comparison to amino acid sequences of other plant myrosinases.

Approximately 2×10^5 recombinant plaques were transferred to a Hybond-N* membrane (Amersham Pharmacia Biotech) and screened by hybridization with the PCR product described above. The probe was labeled and detected using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim). Hybridization was performed at 60°C in the hybridization buffer; 5× SSC (1× SSC: 15 mM sodium citrate buffer containing 150 mM NaCl), 0.1% (w/v) N-laurylsarcosine, 1% (w/v) blocking reagent, and 0.02% (w/v) SDS. The filters were washed twice in 2× SSC-0.1% (w/v) SDS for 5 min at room temperature, then twice in 0.1% SSC-0.1% (w/v) SDS for 15 min at 60°C. Positive plaques were isolated and converted into the pBluescript (SK) vector (Stratagene) by an in vivo excision according to the manufacturer’s instructions.

Nucleotide sequencing was performed using an SequiTherm EXCEL II Long-Read Premix DNA Sequencing Kit-LC (Epigen Tech- nologies) on a DNA sequencer (Model 4000L, Aloka), after the generation of a series of deleted plasmids. The nucleotide and amino acid sequences were analyzed by GENETYX-MAC software (Software Development). A computer search of DDBJ databases was done with the BLAST programs (Altschul et al. 1990).

**Northern blot analysis**

Total RNA was isolated according to the phenol/SDS method (Hussey and Hunsperger 1996). Each RNA sample (10 μg for seedlings and 20 μg for mature plants) was fractionated on a formaldehyde-agarose gel and blotted onto a Hybond-N* membrane (Amersham Pharmacia Biotech). The membranes were hybridized with a DIG-labeled RMB1 cDNA as a probe at 68°C in the hybridization buffer. After hybridization, the blots were washed with 2× SSC-0.1% (w/v) SDS at 55°C for 5 min at room temperature and 0.1× SSC-0.1% (w/v) SDS for 15 min at 68°C. Positive signals were visualized by the methods in the instruction manuals (Boehringer Mannheim).

**Assay of myrosinase activity**

Myrosinase activity was measured by the method to determine glucose liberation (James and Rossiter 1991) with slight modification. Fresh tissue (0.5 g for seedlings and 5 g for mature plants) was weighed and ground in a mortar with 3 volumes of reaction buffer (100 mM potassium phosphate buffer pH 7.0) containing 5 mM dithiothreitol on ice. The homogenate was centrifuged for 15 min at 12,000×g. For the assay of mature plants, the supernatant was adjusted to 80% saturation with ammonium sulfate and proteins were collected by centrifugation. The precipitate was dissolved in a small volume of the reaction buffer and subjected to a NAP-5 column (Amersham Pharmacia Biotech) equilibrated with the reaction buffer. To measure the activity in seedlings, the supernatant from homog enate was directly applied to the NAP-5 column. The eluate of the column was used for myrosinase assay. The assay mixture (950 μl) containing the reaction buffer and 100 μM sinigrin (allylglucosinolate, Sigma) was preincubated for 5 min at 37°C. The reaction was started by addition of the enzyme extract (50 μl) to the mixture and stopped by boiling for 5 min after appropriate incubation periods at 37°C. After centrifugation at 12,000×g for 15 min, the content of glucose in the supernatant was determined using an enzymatic Glucose (GO) Assay Kit (Sigma). One unit (U) of myrosinase activity was defined as the enzymatic hydrolysis of 1 nmol sinigrin min⁻¹. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

**Determination of glucosinolate content**

Since plants contain various kinds of glucosinolates (Fenwick et al. 1983), measurement of total glucosinolates was necessary to analyze all types of glucosinolates. Total glucosinolates were quantified by the method described previously (Carlson et al. 1987) with slight modification. Fresh tissue (0.2 g for seedlings and 5 g for mature plants) was extracted with 10 volumes of boiling ethanol. The extract was labeled and detected using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim). Hybridization was performed at 60°C in the hybridization buffer; 5× SSC (1× SSC: 15 mM sodium citrate buffer containing 150 mM NaCl), 0.1% (w/v) N-laurylsarcosine, 1% (w/v) blocking reagent, and 0.02% (w/v) SDS. The filters were washed twice in 2× SSC-0.1% (w/v) SDS for 5 min at room temperature, then twice in 0.1% SSC-0.1% (w/v) SDS for 15 min at 60°C. Positive plaques were isolated and converted into the pBluescript (SK) vector (Stratagene) by an in vivo excision according to the manufacturer’s instructions.

Nucleotide sequencing was performed using an SequiTherm EXCEL II Long-Read Premix DNA Sequencing Kit-LC (Epigen Technologies) on a DNA sequencer (Model 4000L, Aloka), after the generation of a series of deleted plasmids. The nucleotide and amino acid sequences were analyzed by GENETYX-MAC software (Software Development). A computer search of DDBJ databases was done with the BLAST programs (Altschul et al. 1990).
was evaporated and the residue was taken up in 10 ml H₂O. The aqueous solution was divided into two equal volumes. One sample was directly applied to a 4 ml column of DEAE Sephadex A-25 (acetate form) and the other sample was supplemented with 2 μmol sinigrin before the column operation in order to calculate the yield of glucosinolates. The column was washed with 15 ml H₂O and the glucosinolates binding to the resin were digested with 100 U myrosinase for 2 h at 37°C that was partially purified as described by Carlson et al. (1981). The amount of glucose released from the immobilized glucosinolates was determined using a Glucose (GO) Assay Kit (Sigma) and the content of total glucosinolates extracted was determined in consideration of the yield.

Tissue printing

Tissue print hybridization for myrosinase gene expression was adapted from the method of Song et al. (1993). The root of mature plants was cut transversely at the middle. The freshly cut surface was slightly blotted with Kimwipes and immediately pressed against a Hybond-N + membrane (Amersham Pharmacia Biotech) supported with three layers of 3 MM filter paper (Whatman) for 15 s. At least three prints were prepared by repeated cutting close to the first cut from the same plant. The RNA was fixed to the filter by UV crosslinking for 3 min. The filter was washed with 0.2× SSC and 1% (w/v) SDS at 70°C for 4 h prior to hybridization. The probes were sense and antisense DIG-labeled RNA transcribed from a 740 bp PCR product described above with T7 and SP6 RNA polymerase (DIG RNA Labeling Kit, Boehringer Mannheim). The template was pGEM-T Easy vector (Promega) inserted with the PCR fragment. Specific hybridization of the antisense RNA probe to myrosinase mRNAs in total RNA from the radish root was checked by Northern analysis. Although both antisense and sense probes were weakly hybridized to rRNAs, this nonspecific hybridization could be reduced under high stringency. Hybridization, washing, and detection were carried out by the same procedure as for Northern blot analysis except for the hybridization temperature at 70°C. A filter was prepared exclusively to visualize RNA blotted on the membrane and stained with 0.03% methylene blue solution described by Wilkinson et al. (1991).

Tissue printing for myrosinase activity was performed as follows. A PVDF membrane (ATTO) was pretreated with methanol then a printing buffer (5 mM potassium phosphate buffer pH 7.0). The transverse cut end of radish root was lightly blotted with Kimwipes and quickly printed onto the membrane supported by Whatman 3 MM paper for 15 s. Three prints were prepared from the same plant. After the wash with printing buffer for 30 min at room temperature, the filter was incubated in a reaction solution containing 5 mM potassium phosphate buffer pH 7.0, 1.2 mg ml⁻¹ glucose oxidase (Sigma), 50 μg ml⁻¹ horseradish peroxidase (Wako), 50 μg ml⁻¹ O-dianisidine (ICN), and 400 μg ml⁻¹ sinigrin (Sigma) approximately for 1 h until the signals appeared at room temperature. As a control, another filter was incubated in the reaction solution without sinigrin for the same reaction period. Filters were washed twice with H₂O for 30 min to stop the reaction. Protein on the print was stained with Coomassie Brilliant Blue R-250 (Fluka).

To identify the histochemical position of signals in tissue printings, freehand slices were directly stained with 0.05% toluidine blue O (Sigma).

Results

Cloning of radish myrosinase

Myrosinase cDNA clones were isolated from radish seedlings cDNA library. To screen the library, the PCR fragment of radish myrosinase was prepared. Poly(A)+ RNA from 5-day-old seedlings was used as a template of RT-PCR. We designed three kinds of primers for nested PCR. The sequences of primers 1, 2 and 3 appear in materials and methods. The target region of the present PCR was the nucleotide sequence encoding a portion of N terminus in myrosinase and the fragment sizes predicted were 740 bp (1st PCR with the combination of primers 1 and 3) and 420 bp (2nd PCR with primers 1 and 2). First PCR amplified a major fragment of 740 bp and several minorities. The fragments were re-amplified by the second PCR and a single fragment of 420 bp was obtained. After subcloning the PCR products into the plasmid vector, 16 individual clones were sequenced and could be separated into two homologous groups. Each clone was similar to the expected region of myrosinase reported previously (Rask et al. 2000). The cDNA library was screened by one of the PCR products as a probe and 18 positive clones were isolated. Partial sequence and restriction map suggested that these clones could be classified into two groups (8 and 10 clones). A full-length clone in each group
Cloning and expression of radish myrosinase 1105

was sequenced and named RMB1 and RMB2 for radish myrosinase B types 1 and 2. RMB1 was 1,810 bp in length and the open reading frame (ORF) started at nucleotide 9 and ended at nucleotide 1,655, including the stop codon. The length of RMB2 was 1,807 bp and ORF was from nucleotide 13 to 1,653. Each ORF could encode a polypeptide containing 548 amino acids for RMB1 and 546 amino acids for RMB2. The predicted molecular weight was 62,875 and 62,209 in RMB1 and RMB2, respectively. The alignment of the amino acid sequences is shown in Fig 2. The two clones were quite similar to each other and the homology at the amino acid level was 93%. RMB1 and RMB2 contained family 1 O-glycosidases motifs (Henrissat 1991). Conserved amino acids for glucosidic bond formation, glucose ring recognition and aglycon pocket (Burmeister et al. 1997) also existed in both clones. The numbers of putative N-glycosylation sites were 4 and 5 in RMB1 and RMB2, respectively.

Amino acid sequence similarities between radish myrosi-
nases and those from other plants are shown in Table 1. Myro-

<table>
<thead>
<tr>
<th>Myrosinase (subtype)</th>
<th>RMB2</th>
<th>Myr1 (MA)</th>
<th>MYRA (MA)</th>
<th>MYR1 (MB)</th>
<th>MYR (MB)</th>
<th>MB3 (MC)</th>
<th>MYR2 (TGG)</th>
<th>tgg1 (TGG)</th>
<th>tgg2 (TGG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMB1</td>
<td>93</td>
<td>69</td>
<td>65</td>
<td>94</td>
<td>90</td>
<td>88</td>
<td>76</td>
<td>70</td>
<td>66</td>
</tr>
<tr>
<td>RMB2</td>
<td>70</td>
<td>67</td>
<td>94</td>
<td>91</td>
<td>89</td>
<td>77</td>
<td>71</td>
<td>71</td>
<td>67</td>
</tr>
</tbody>
</table>

RMB1 and RMB2 from *Raphanus sativus* (present study). Myr1 (Z21977), MYR1 (X60214) and MYR2 (X79080) from *Brassica napus*. MYR (AJ223494) from *B. juncea*. MYRA (DDB: 1MYR) and MB3 (X59879) from *Sinapis alba*. tgg1 (X79194) and tgg2 (X79195) from *Arabidopsis thaliana*. MA, MB, MC and TGG meant myrosinase A, B, C and *Arabidopsis* type, respectively. Values were expressed as the percentage of deduced amino acid sequence identity.

Distribution of myrosinase-gluconolactone system in radish

Myrosinase gene expression, enzyme activity and total glucosinolates content were detected in different tissues of radish. The cotyledon was the organ that showed the largest myrosinase activity and content of total glucosinolates, calculated as per g FW, followed by the hypocotyl and root (Fig. 3). The hybridization signals in Northern analysis indicated that the highest expression was observed in the hypocotyl and the lowest expression in the root. However, computer-graphic analysis of the NIH Image revealed that if the signal intensity was shown

Fig. 3 Myrosinase mRNAs accumulation, enzyme activity, total glucosinolates, and fresh weight in different organs of radish seedlings. A, B, C, and D show cotyledon, hypocotyl, root, and whole seedlings, respectively. For Northern analysis, total RNA (10 μg) from cotyledon, hypocotyl, root, and whole seedlings was fractionated using an agarose gel and hybridized using an RMB1 clone. Myrosinase activity and the content of total glucosinolates in each part were determined as described in Materials and Methods. In myrosinase activity, total glucosinolates and fresh weight, average values of three replicates are shown with bars as SE.

Fig. 4 Myrosinase mRNAs accumulation, enzyme activity, total glucosinolates, and fresh weight in different organs of mature radish. A, B, C, D, E, F, and G exhibited leaf, root, root peel, peeled root, and top, middle, and bottom of root, respectively, as described in Materials and Methods. For Northern analysis, each total RNA (20 μg) from leaf, root, root peel, peeled root, and top, middle, and bottom of root was fractionated by an agarose gel and RMB1 clone was used for hybridization. Myrosinase activity and the content of total glucosinolates in each part were determined as described in Materials and Methods. In myrosinase activity, total glucosinolates and fresh weight, average values of three replicates are shown by bars as SE.
Cloning and expression of radish myrosinase

as per g FW, the expression in the cotyledon was approximately double that in the hypocotyl. Thus, myrosinase expression and glucosinolate accumulation in radish seedlings occurred mainly in the cotyledon.

The values of myrosinase gene expression, gene product and glucosinolate were higher in the root than in the leaf (Fig. 4). In the root, they were mainly detected in the peel. The peel had 6-fold and 3-fold higher myrosinase activity and glucosinolate content than the peeled root, respectively. According to root portion, the myrosinase activity was in the order of bottom > middle > top and gene expression bottom > middle > top. It was supposed that the difference of myrosinase activity in peeling could reflect activity of each part, because the peel from the bottom of the root contained higher enzymatic activity than that from top or middle of the root (data not shown). Although there were some differences in the details, the tissue-distribution pattern of the three factors, myrosinase transcript accumulation, enzyme activity and glucosinolate content, were similar throughout the plant.

**Tissue printing for gene expression and enzyme activity of myrosinase**

The Northern analysis and measurement of enzyme activity in different tissues suggested that myrosinase gene expression and enzymatic activity were located mainly in the roots. For further analyses of myrosinase distribution in the root, tissue print for gene expression were made and enzyme activity measured. The results for a transverse cut is shown in Fig. 5. The tissue print technique is a rapid and convenient method to localize proteins and mRNAs in plant tissues (Song et al. 1993). Although many molecules have been detected by this method (Varner and Ye 1994), tissue printing for enzymes involved in secondary metabolism has been reported, such as peroxidases (Peyrano et al. 1996) and O-methyltransferase (Bugos et al. 1991) associated with lignification. Here, we applied tissue print hybridization to detect myrosinase mRNAs and also developed a novel method for tissue printing to visualize myrosinase activity. In tissue print hybridization, specific signals were detected especially in the peripheral part of the root only when an antisense RNA probe was used (Fig. 5A, B). A similar image was obtained in tissue printing enzyme assay when sinigrin was supplemented to the reaction system (positive reaction, Fig. 5D) but no signals were detected in the control (Fig. 5E). RNA and protein were stained with methylene blue (Fig. 5C) and Coomassie Brilliant Blue (Fig. 5F), respectively. Both RNA and protein were present in the whole area of

---

**Fig. 5** Tissue printing for myrosinase mRNAs and enzyme activity in radish root.prints were prepared from the transversely cut surfaces of root. Tissue print techniques were applied to detect myrosinase transcripts (A–C) and myrosinase activity (D–F). DIG-labeled antisense and sense RNA probes transcribed from RMB1 cDNA clone were used to hybridize with myrosinase RNAs blotted onto nylon membrane (A, antisense hybridization; B, sense hybridization). RNA on the filter was stained with methylene blue (C). Enzymatic activity of myrosinase immobilized on PVDF membrane was detected by the sinigrin-glucose oxidase-peroxidase system described in Materials and Methods (D, full reaction with sinigrin; E, control reaction without sinigrin). Protein on the filter was stained with Coomassie Brilliant Blue (F). A crude slice (G) was stained with toluidine blue (H) to visualize histochemical structures, such as epidermis, cortex, vascular cambium, and parenchyma. Typical dotted signals that frequently appeared in tissue printings are indicated by arrowheads.
the cut surface, although staining was slightly inhomogeneous.

In order to determine the histochemical location corresponding to the signals in tissue printings, crude slice of root (Fig. 5G) was stained with toluidine blue O (Fig. 5H). Vascular cambium was present at 1–2 mm inside of the epidermis. The internal space of root was filled with parenchyma cells and vascular bundles were scattered in the parenchyma. Both mRNA and enzyme activity of myrosinase was detected intensively in epidermis and vascular cambium. There were also weak signals in the cortex. Frequently, a dot shape was characteristic of signals that appeared on the vascular cambium in both printings (arrowheads in Fig. 5). Myrosinase distributions in epidermis and vascular cambium were also observed when tissue printings were prepared from a vertically cut root (data not shown).

In tissue printing, it was difficult to localize the exact position of myrosinase mRNA and activity into specific site of tissue at the cellular level, since the information on positive signals was obtained from an indirect image. Thus, we could not determine whether myrosinase-expressing cells corresponded to cells that possessed myrosinase activity or which types of cells contained myrosinase specifically.

**Discussion**

Although a short EST clone (AF051118) for radish myrosinase has been reported previously, no sequences for full-length ORF have appeared in the databases. We isolated two cDNA clones of myrosinase, RMB1 and RMB2, from radish (*R. sativus*). These clones were very similar with 91% and 93% homology at the nucleic acid and amino acid level, respectively. Since the numbers of positive clones were 8 (RMB1) and 10 (RMB2), each gene seemed to be expressed to almost the same degree. From these data, it was presumed that RMB1 and RMB2 were alleles from parents or homologs of a multigene family. RMB2 matched well with the EST (AF051118) in sequence. Recently, Shikita et al. (1999) purified myrosinase protein from 8-day-old radish seedlings and obtained two isoforms indivisible by chromatography. The isoforms had a molecular mass of 61 kDa and 62 kDa on SDS-PAGE analysis and were assumed to form a heterodimer. In the present study, RMB1 and RMB2 have predicted molecular masses of 62.9 kDa and 62.2 kDa, respectively. These results indicated that RMB1 and RMB2 could encode each isoform purified by Shikita et al. (1999) and each gene product might form a heterodimer. Exact comparison seems to be difficult because cultivars were different, daikon (Japanese radish) for protein purification and general radish for cDNA cloning, although both cultivars were *R. sativus* L. (Carlson et al. 1985).

A homology search of DDBJ databases by BLAST and a calculation of similarity at an amino acid level showed that RMB1 and RMB2 were included in MB (Table 1). MB genes are expressed overwhelmingly in the whole life cycle but MA and MC were expressed only in developing seeds in *B. napus* (Lenman et al. 1993, Falk et al. 1995). Our finding, that only MBs were isolated from radish seedlings, suggested that MBs might be the most expressed of the genes of radish as in *B. napus*. The evolutionary tree among MBs made by UPGMA program (GENETYX-MAC software, Software Development) indicated that the phylogenetically closest myrosinases to MYR1 of *B. napus* were RMBs followed by MYR of *B. juncea* and MB3 of *S. alba* (data not shown). It was interesting that *R. sativus* could be categorized into *Brassica* in phylogenetical association of myrosinase sequences.

Tissue distribution of myrosinase gene expression and enzyme activity have been well studied in embryos and seedlings of Brassicaceae. Myrosin cells, stained with Millon’s reagent and lactophenol-aniline blue (Phelan and Vaughan 1980), are specialized cells containing myrosinase (Kelly et al. 1998). Myrosin cells are observed in the cotyledons and hypocotyl of the embryo, gradually decreasing during seed imbibition, and completely disappeared within a week after sowing (Bones and Iversen 1985). Immunocytochemical and molecular biological studies on myrosinase distribution in seedlings also revealed that the cotyledon and hypocotyl were main myrosinase-containing organs in *B. napus* (Bones et al. 1991, James and Rossiter 1991), *S. alba* (Xue et al. 1992) and *A. thaliana* (Xue et al. 1995). In radish seedlings, similar localization of myrosinase in gene expression and enzymatic activity was observed (Fig. 3).

On the other hand, there was little information about the distribution of myrosinase in mature plants. Bones (1990) described that the major myrosinase-containing organ in *B. napus* was the root which showed 10- to 100-fold higher myrosinase activity than the stem or leaf. As shown in Fig. 4 like *B. napus*, radish also exhibited marked myrosinase activity in the root but low activity in the leaf. The high myrosinase activity in the root seemed to be supported by strong gene expression. In addition, it was shown that the greater part of expression and activity in the root was located in the peel especially epidermis and vascular cambium (Fig. 5). In the case of *B. napus*, the anti-myrosinase antibody reacted with the cells in both periphery of radicle and vascular tissue of hypocotyl in the seed embryo imibed for 4 h (Bones et al. 1991). Myrosinase expression at periphery and vascular tissue was assumed to be a common property in not only the embryo but also the mature plant.

We detected myrosinase gene expression, enzyme activity and glucosinolate from various tissues of radish. The quantitative analysis of glucosinolates in different tissues implied a tendency that high accumulation of glucosinolates occurred in the tissues where myrosinase was expressed abundantly (Fig. 3, 4). This suggested that myrosinase and glucosinolates were arranged genetically in a restricted area in order to make myrosinase-glucosinolate system function efficiently. Although it is already known that high yields of glucosinolates were extracted from the peel rather than peeled root of root vegetables such as radish (Carlson et al. 1985) or turnip (Carlson et al. 1981), the
distribution of myrosinase in the root has not been well understood. We reported that the root peel was the main accumulation tissue of myrosinase-glucosinolate system in radish. The putative role of myrosinase-glucosinolate system in the peel was to protect the root, a significant sink organ, from the attack of insects, pathogens or mammals in soil.

References


(Received May 12, 2000; Accepted July 20, 2000)