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A young root-specific gene (*ArMY2*) from horseradish encoding a MYR II myrosinase with kinetic preference for the root-specific glucosinolate gluconasturtiin

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ABSTRACT

The pungent taste of horseradish is caused by isothiocyanates which are released from glucosinolates by myrosinases. These enzymes are encoded by genes belonging to one of two subfamilies, termed MYR I and MYR II, respectively. A MYR II-type myrosinase gene was identified for the first time in horseradish. The gene termed *ArMY2* was only expressed in young roots. A full-length cDNA encoding a myrosinase termed ArMy2 was isolated and heterologously expressed in *Pichia pastoris*. The recombinant His-tagged enzyme was characterized biochemically. Substrate affinity was 5 times higher towards gluconasturtiin than towards sinigrin. Gluconasturtiin was found to be the most abundant glucosinolate in young horseradish roots while sinigrin dominated in storage roots and leaves. This indicates that a specialized glucosinolate–myrosinase defense system might be active in young roots.

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Introduction

Horseradish is a perennial Brassicaceae crop plant which is cultivated throughout the temperate climatic zones. Its storage roots are used as a condiment in human diet because of their pungent aromatic taste caused by isothiocyanates. Evidence for several health-beneficial effects of isothiocyanates is accumulating, including anti-carcinogenesis and protection of the central nervous and the cardiovascular system (Dinkova-Kostova and Kostov, 2012). Isothiocyanates are released from sulfur-containing glycosides (glucosinolates) by the enzyme myrosinase (EC 3.2.1.147) and subsequent Lossen-type rearrangement of the unstable intermediate (Fig. 1). The most abundant glucosinolate found in storage roots and leaves of horseradish is sinigrin which yields allyl isothiocyanate upon hydrolysis (Li and Kushad, 2004). The glucosinolate–myrosinase system is supposed to exist in all members of the Brassicaceae as well as in many other Brassicales (Mithen et al., 2010). Operating as a possible defense mechanism against herbivores and pathogens, myrosinase and its substrates are stored separate from each other until cellular compartmentalization is disintegrated (Bones and Rossiter, 1996; Halkier and Gershenzon, 2006).

Myrosinases are *S*-glucosidases that form a distinct cluster in the glycosyl hydrolase family 1 (Henrissat et al., 1995). They have most likely evolved from an *O*-glucosidase ancestor (Rask et al., 2000). Plant myrosinases *sensu stricto* are encoded by genes belonging to one of two subfamilies termed MYR I and MYR II, respectively (Wang et al., 2009a). Several MYR I and few MYR II myrosinase isoenzymes have been described from various plants. All of them are glycosylated and the carbohydrate side chains contribute 9–23% of the molecular mass of a myrosinase (Zhou et al., 2012). Glycosylation was thought to be essential for myrosinase activity (Wang et al., 2009a) until a recent finding showed that deglycosylated myrosinase is also catalytically active (Zhou et al., 2012). Most plant myrosinases can be activated by L-ascorbic acid (Shikita et al., 1999).

A myrosinase termed ArMy1 was isolated from horseradish roots and the purified enzyme partially sequenced (Li and Kushad, 2005). The peptide sequences reported indicate a close relationship to the MYR I myrosinases encoded by the genes AtTGG1 (Acc. Nr.: NM_180746.2), AtTGG2 (NP_001031940.1) in *Arabidopsis thaliana*. *A. thaliana* has six typical myrosinase genes (ATTG1 to ATTG6; Andersson et al., 2009) and at least one atypical myrosinase gene (PEN2; Bednarek et al., 2009). AtTGG1, AtTGG2 and AtTGG3 reside on chromosome 5, whereas AtTGG4 (NM_103653.2), AtTGG5 (NM_104025.3) and AtTGG6 reside on chromosome 1 (Andersson et al., 2009). AtTGG1 and AtTGG2 are expressed in above-ground tissues whereas AtTGG4 and AtTGG5, which belong to the MYR II

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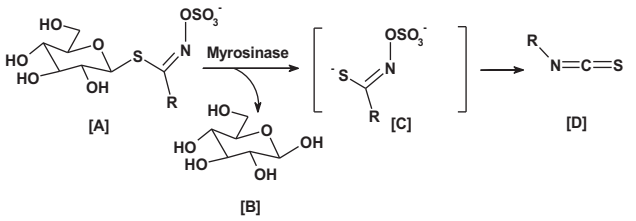

Fig. 1. Myrosinase-catalyzed deglycosylation of a glucosinolate (A). β-D-glucose (B) and an unstable intermediate (C) are released. C undergoes rearrangements to finally yield an isothiocyanate (D).

myrosinases, are expressed in the roots only (Andersson et al., 2009). AtTGG3 and AtTGG6 are considered as pseudogenes (Wang et al., 2009b; Xu et al., 2004). The others have been demonstrated to code for active myrosinases (Andersson et al., 2009).

*ArMY*1 (Acc. Nr.: AY822710) was the first and so far only myrosinase gene characterized in horseradish. It is expressed in storage roots as well as in leaves (Hara et al., 2008; Li et al., 2007). It is a close homolog of the *A. thaliana* MYR I genes. Until now, *A. thaliana* is the only Brassicaceae demonstrated to possess functional MYR I and MYR II myrosinases (Andersson et al., 2009; Zhou et al., 2012). The recent identification of MYR II genes in *Carica papaya* (Nong et al., 2010; Wang et al., 2009a), which are closely related to the *A. thaliana* MYR II genes, and the assumption that another myrosinase gene subfamily may be present in roots prompted us to aim at the identification of a MYR II gene in horseradish roots. For the first time, we describe the molecular cloning of a MYR II myrosinase gene in horseradish and its heterologous expression in *Pichia pastoris*. The gene, termed *ArMY*2, encodes a functional myrosinase and was expressed specifically in young roots of horseradish.

Results and discussion

Isolation of the ArMY2 gene from young root tissue of horseradish

The primers ArMy2_786 dir and ArMy2_786 rev (Table 1) deduced from *A. thaliana* TGG4 (Acc. Nr.: NM_103653.2) were used to identify a MYR II gene in horseradish. Genomic DNA isolated from horseradish leaves served as the template. After identification the primers SSP1, SSP2 and SSP3 were designed and used for TAIL-PCR. In this way a large DNA fragment adjacent 5′ to the initial primers was recovered. It included the start codon of a putative horseradish MYR II gene and its promotor region (Acc. Nr.: KF761296). AtTGG4 and AtTGG5 have been reported to be expressed specifically in roots (Andersson et al., 2009; Kissen et al., 2009). Therefore, initially fully developed horseradish roots were used to pursue the isolation of a putative MYR II cDNA. Since this attempt failed, axenic root cultures were established. Meristematic tissue of the shoot apex was used as the starting material to initiate axenic organ cultures since horseradish seeds are not commercially available (Górecka, 1992). Finally, mRNA prepared from thin young roots grown *in vitro* on agar-solidified medium was successfully used for the isolation of a 1531-nucleotide full-length cDNA (Acc. Nr.: JN638573.1) encoding a putative MYR II myrosinase. The respective gene was termed *ArMY*2.

According to calculations with BioEdit 7.2 (Hall, 1999) the protein encoded by *ArMY*2 (EC 3.2.1.147; Fig. 2A), shares only a low sequence identity of 48% with ArMy1 (Li et al., 2007), a member of the MYR I subfamily. On the other hand, ArMy2 sequence identity with the MYR II myrosinases of *A. thaliana* with ca. 90% is higher then expected. A screening of the Phytozome plant genome database (www.phytozome.net) revealed further hypothetical MYR II homologs in *Thellungiella halophila*, *Brassica rapa* and *Capsella rubella*. Using the available data an updated phylogenetic tree of the myrosinase gene family (Nong et al., 2010; Wang et al., 2009a) was constructed. The phylogenetic tree in Fig. 3 supports the concept of two myrosinase subfamilies. The MA, MB and MC myrosinase genes described (Rask et al., 2000) all belong to the MYR I cluster. ArMy1 and ArMy2 cluster with MYR I myrosinases and MYR II myrosinases of *A. thaliana*, respectively. The *PEN*2 gene (At2g44490) of *A. thaliana* encodes a myrosinase which cleaves indolic glucosinolates. It is supposed to be involved in a specific defense mechanism against fungal infections (Bednarek et al., 2009). PEN2 is not a member of the classic myrosinases (Nong et al., 2010) and lacks several motifs typical for myrosinases (Zhou et al., 2012). It was therefore not considered in the phylogenetic tree in Fig. 3.

Protein sequence analysis

ArMy2 consisted of 510 amino acids with a calculated *M*r. of 57.5 kDa. It was compared with myrosinases of *A. thaliana* and other Brassicaceae using ClustalW multiple alignment (Thomson et al., 1997) and SignalP 4.1 analysis (Petersen et al., 2011) (Fig. 2B). *A. thaliana* myrosinases possess signal peptides which are necessary for allowing the enzymes to enter the endoplasmic reticulum. This is a prerequisite for subsequent glycosylation, a typical feature of plant myrosinases. ArMy2 has a signal peptide of 22 residues (Fig. 2A) and may thus be regarded as a pre-protein of the mature ArMy2 with a calculated *M*r. of 55.2 kDa.

3D protein structure homology modelling of ArMy2 (SWISS-MODEL; Kiefer et al., 2009) using the crystal structure of *Sinapis alba* MYR1 (Burmeister et al., 1997) as the template, showed identical topology regarding an amino acid side chain known to interact with Zn^{2+} and the sugar moiety of the glucosinolate (Fig. 4). Multiple alignments of known myrosinases from Brassicaceae show characteristic differences between MYR I and MYR II enzymes, *e.g.*, in the aglycone-binding site (positions 220, 224, 402; Fig. 2B) and in the conserved MYR I motif 1 (Zhou et al., 2012)

Table 1
Primers for PCR (expression analysis and cloning).

Name		Sequence
Actin	fw	ATTCAGGATGCCCAGAAGTCTTGTT
	rev	GGAGATCCACATCTGCTGGAATGT
ArMy2_786	dir	TGCTGCACATAGAGCACTTAATG
	rev	GGCATTCTATCTCCTACCATCTC
ArMy2Clon	dir	GGGCCTAGGACCATGGCAATTCCCAAAGCTCACTAC
	rev	GGGGCGGCCGCCTAATGGTGATGGTGATGGTGTTTT-GCGAGGAACTTAGAGAACC
My2	dir	ATGTCAATTCCCAAAGCTCACTAC
	rev	TTATTTTGCGAGGAACTTAGAGAACC
SSP1		TATCCACCGGGACGGTGCATCGACTGTG
SSP2		ACGATGGACTCCAGTCCGGCCGCTCCCATTCTCGTC-CACACCTCCAATCAG
SSP3		ACGAATCACAAGCAAGGTCTCCTGAACTTGG

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