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Characterisation of *Brassica napus* L. metallothionein genes (*BnMTs*) expression in organs and during seed germination

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Abstract

The aim of this work was to identify rape metallotionein genes belonging to all four types of plant MTs and study their expression pattern in rape's organs and during seed imbibition. Using EST sequences found in NCBI database we have identified four metallothionein genes, *BnMT1-4*, from *Brassica napus* L. Analysis of their predicted amino acid sequences confirmed the presence of numerous cysteine residues confirming that these genes encode plant metallothioneins. Both cloned MT nucleotide and amino acid sequences share the most homology with MT gene sequences from the *Brassicaceae* family. The *BnMT1*, *BnMT2* and *BnMT4* genes harbour one intron, whereas the *BnMT3* gene contains two. Examining MT expression by sqRT-PCR in rape cotyledons, leaves, flowers, roots, and hypocotyls demonstrated that *BnMT1* is expressed in each organ except the flower. *BnMT2* and *BnMT3* transcripts are present in all studied organs. Five-day-old cotyledons exhibited the strongest MT expression, whereas mature organs exhibited lower or approximately similar levels of expression. *BnMT4* expression was not observed in the organs; however, it was observed in dry and germinating rape seeds. Additionally, *BnMT1* and *BnMT2* are expressed both in dry and germinating seeds. *BnMT3* transcripts were not observed in dry seeds but were apparent at the 24th hour of imbibition, During the next 12 hours the expression level of *BnMT3* increases approximately 6-fold and lasts through the 5th day, when the seedling develops. The results suggest that *BnMT4* may regulate germination processes. Organ-specific expression of rape MTs was demonstrated, suggesting that these genes play significant roles in embryogenesis, pollen germination, fruit ripening, senescence and thus may potentiate proper plant development.

Keywords: canola, metallothionein, identification of sequence, gene expression.
Abbreviations: Cys - cysteine; dbEST-database for expressed sequence tags; EDTA - ethylenediaminetetraacetate; MT - metallothionein; MMLV-RT - Moloney murine leukaemia virus reverse transcriptase; sqRT-PCR - semi-quantitative reverse transcriptase polymerase chain reaction.

Introduction

Plant metallothioneins (MTs) are small, nuclear-encoded proteins that bind heavy metals via thiol groups of cysteine residues. The first plant MT was isolated from mature *Triticum aestivum* germs and, due to the high Cys content, was marked as E_C (Lane et al., 1987). The majority of MTs from both monocotyledonous plants, including maize (Chevalier et al., 1995), wheat (Kawashima et al., 1992) and rice (Hsieh et al., 1995), and dicotyledonous plants, such as tomato (Whitelaw et al., 1997), soybean (Kawashima et al., 1991), peas (Evans et al., 1990), and cotton (Hudspeth et al., 1996), were identified through cDNA cloning. Genes encoding MT-like proteins have been identified in *Brassicaceae* plants such as *Arabidopsis thaliana* (Zhou and Goldsbrough, 1995), *Brassica rapa* (Ahn et al., 2012), *Brassica napus* (Buchanan-Wollaston, 1994), *Brassica juncea* (Zhigang et al., 2006), and *Thlaspi caerulescens* (van de Mortel et al., 2006). MT genes have also been cloned from the gymnosperm Douglas fir (Chatthai et al., 1997) and marine algae *Fucus vesiculosus* (Morris et al., 1999); however, these genes exhibit dissimilar structures. Plant MTs, unlike animal MTs, are divergent both in structure and expression regulation. Classification of plant MTs based on sequence similarities and phylogenetic relationships divides them into four subfamilies, p1-p3 (type1-3) and pec (type 4) (Cobbett and Goldsbrough, 2002; Freisinger, 2008; Hassinen et al., 2011). Several MTs differ in the number of amino acids (from 45 to 87 aa), the number of Cys-rich domains (two or three), the total content of Cys residues (from 10 to 17), and the length of the spacer regions between Cys-rich domains (variable) (Hassinen et al., 2011). MTs are characterised by a pattern of Cys arrangement within two domains. p1 MTs harbour Cys-Xaa-Cys clusters exclusively, whereas p2 MTs harbour Cys-Cys, Cys-Xaa-Cys and Cys-Xaa-Xaa-Cys clusters within N-terminal domains. Only a subset of Cys residues are contained within Cys-Xaa-Cys motives in p3 MTs. pec MTs harbour three domains rich in Cys residues partially grouped in Cys-Xaa-Cys clusters (Cobbett and Goldsbrough, 2002; Hassinen et al., 2011). MT proteins are responsible for maintaining metal ion homeostasis due to their preferential binding of heavy metal ions, particularly those of d electron configuration (Freisinger, 2008). MTs have been shown to play important functions in detoxifying heavy metals and protecting against oxidative stress in plants (Lee at al., 2004; Mir et al., 2004; Miller et al., 2010; Hrynkiewicz et al., 2012). The role of plant MTs in metal tolerance was first verified through complementation of yeast mutants (Zhou and Goldsbrough, 1994). MTs bind Cu, Cd and Zn ions (reviewed by Hassinen et al., 2011; Freisinger, 2008; Domènech et al., 2006). Zimeri et al. (2005)), showed that *A. thaliana* knock-down lines with reduced *AtMT1a* and *AtMT1*c levels

Table 1. The characteristics of cloned MT sequences in *B. napus*.

Gene and NCBI accession number	Intron/s length [bp]	Amino acid length [aa]	Cysteine residue number	Cysteine sequence pattern	Length of spacer between cysteine domains [aa]	Gene/s with highest homology and percent of identical aa
BnMT1 JX035784.1	133	45	13 (6 + 7)	C-X-C	10	AtMT1 (NP_172240) 96%
BnMT2 JX103200.1	102	80	14 (8 + 6)	C-C, C-X-C, C-X-X-C	42	BrMT2 (BAA11388.1), BjMT2 (CAA71803.1) 100%
BnMT3 JX103201.1	129, 77	67	10 (4 +6)	C-X-X-C, C-X-C	33	BjMT3 (BAB85599.1) 99%
BnMT4 JX103202.1	79	86	17 (6 + 6 + 5)	C-X-C	14, 13	AtMT4 (NP_973520.1) 83%

are hypersensitive to Cd and accumulate less As, Cd and Zn in leaves than wild-type plants. In *Populus tremula x tremuloides* grown on metal-contaminated soils, the foliar *MT2b* transcript level was shown to correlate positively with Cd and Zn concentrations (Hassinen et al., 2009). In addition, plant MTs participate in important developmental processes, such as embryogenesis (Reynolds and Crawford, 1996; Chatthai et al., 1997), root development (Yuan et al., 2008), fruit ripening (Itai et al., 2000; Moyle et al., 2005), senescence (Buchanan-Wollaston and Ainsworth, 1997), and pollen germination (Guyon et al., 2000). Both abiotic factors, such as heat shock (Hsieh et al., 1996), wounding (Choi et al., 1996), flooding (Majić et al., 2008), darkness (Chen et al., 2003), and drought (Yang et al., 2009; Miller et al., 2010), and biotic ones, such as viral infection (Choi et al., 1996), the presence of mycorrhizal fungi (Lanfranco et al., 2002) or PGPR bacteria in soil (Hrynkiewicz et al., 2012), influence the changes in MT expression. Other factors, such as salt stress (Mir et al., 2004), sucrose starvation (Chevalier et al., 1995), and phytohormone activity (Reynolds and Crawford, 1996; Itai et al., 2000), also affect MT transcript levels. Several studies have suggested that MT-like protein isoforms differ not only in sequence but also in the functions they perform in specific tissues (Cobbett and Goldsbrough, 2002). Type 1 MT genes are activated more abundantly in roots than in leaves of several plant species, whereas type 2 MT genes are expressed primarily in leaves (Zhou and Goldsbrough, 1995; Hsieh et al., 1996). Type 3 MTs are expressed in leaves or in ripening fruits (Ledger and Gardner, 1994), whereas type 4 MT expression appears to be restricted to developing seeds (White and Rivin, 1995). Isolation of MT cDNA from genes that are induced at various stages of development and contribute to the plant response to different stress factors allows for examining their role in plant development and adaptation to changing environmental conditions. We selected *Brassica napus* L., a crop oil plant cultivated in Europe, Australia, North America, China and the Indian subcontinent, for this study because it can be transformed with foreign DNA using *Agrobacterium*. Moreover, rape is closely related to the well-characterised model plant *Arabidopsis thaliana*, of which numerous mutants were obtained. The aim of this study was to clone and analyse cDNA nucleotide sequences of particular types of *Brassica napus* L. metallothioneins and to determine the pattern of their expression in plant organs and during rape seed germination, which may be a significant factor in crop yield improvement.

Results and Discussion

Identification of rape metallothionein sequences

Genes encoding plant metallothioneins form small families. In the completely sequenced genomes of *A. thaliana* and *O. sativa*, 7 and 11 MT genes were discovered, respectively, and were assigned to four distinct types (Guo et al., 2003; Zhou et al., 2006). Six genes were identified in poplar, two each of types 1, 2 and 3 (Kohler et al., 2004). Three broad bean genes of type 1 and two of type 2 (Foley et al., 1997 and data from NCBI GenBank) and two pea genes of type 1 and one of type 2 have been identified to date (Evans et al., 1990 and data from NCBI GenBank). MT-encoding sequences are short, consistent with the resulting proteins exhibiting molecular masses less than 10 kDa (Cobbett and Goldsbrough, 2002). We used *B. napus* EST sequences homologous to *A. thaliana* MT sequences to design PCR primers with rape cDNA as a template. Four *Brassica napus* L. cDNAs (*BnMT1-4*) were cloned, sequenced and sent to NCBI's GenBank (Table. 1). Their open reading frames are 138, 243, 204 and 261 bp, respectively. The cloned cDNAs are likely full or nearly full-length, as their size is consistent with their respective mRNA sizes deduced from Northern hybridization (data not shown). Their predicted amino acid sequences were analysed by BLASTP. A high similarity between the obtained rape sequences and the MTs of other closely related plants was observed (Table. 1), confirming that metallothioneins are encoded by *BnMT1-4* cDNA sequences. A dendrogram obtained from a phylogenetic analysis of predicted amino acids sequences (Fig. 1) allows for assigning them to particular MT types according to *A. thaliana* sequences. The *B. napus* genome likely contains more than four MT genes; the final number would be possible to establish only after full sequencing. Genomic fragments spanning the coding regions were amplified by PCR and sequenced. The single introns of *BnMT1, BnMT2 and BnMT4* were sequenced, revealing lengths of 133, 102 and 79 bp, respectively. The *BnMT3* sequence harbours two introns of 129 and 77 bp in size (Table. 1). All recognized MTs encoding plant genes are characterised by a discontinuous structure, and introns are localised in preserved positions within each of the gene types (Cobbett and Goldsbrough, 2002). Similar to rape, sequences encoding MT types 1, 2 and 4 in *Arabidopsis thaliana* are separated with one intron, and *At*MT*3* sequences are separated by two introns (Guo et al., 2003). However, in other species, including rice (Chen et al., 1998; Zhou et al., 2006) and *Helianthus tuberosus* (Chang et al., 2004), two introns have been reported in M*T2* genes and in *Ipomoea nil* (G. Dąbrowska, pers. comm.), *Ipomoea batatas* (Chen et al., 2003), cotton (Hudspeth et al., 1996) and rice (Zhou et al., 2006) in genes of MT type 1. Cys residues present in MT sequences are responsible for metal binding. Wheat E_C protein has been shown to bind six divalent metal ions in two separate metal thiolate clusters harbouring 17 cysteine residues (Peroza and Freisinger, 2007). Predicted BnMT amino acids sequences harboured Cys residues arranged in characteristic clusters (Table. 1, Fig. 2). Small differences in

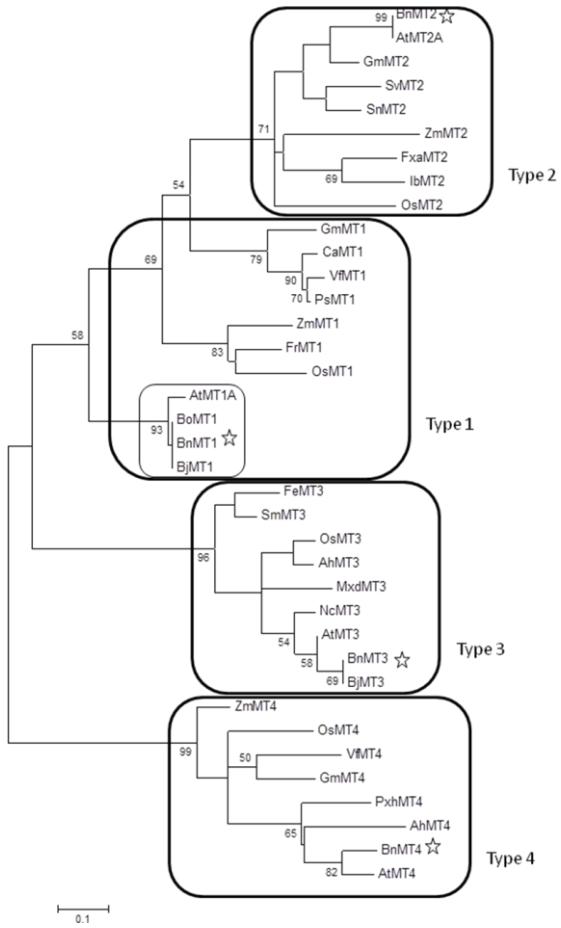

Fig 1. Phylogenetic analysis of plant metallothionein proteins obtained using MEGA4.1. For nodes above or below the bootstrap values greater than 50% are given. Individual types of plant metallothioneins are boxed. The smaller box denotes *Brassicaceae*-specific type 1 MTs. Asterisks identify *B. napus* MT sequences described in this paper. The following amino acid sequences deposited in GeneBank NBCI were used: Ah – *Arachis hypogaea* (acc. no. AAO92264.1-MT3, acc. no. ABG57067.1-MT4), At – *Arabidopsis thaliana* (acc. no. NP_172239.1- MT1, acc. no. NP_187550.1-MT2, acc. no. NP_566509.1-MT3, acc. no. NP_179905.1-MT4), Bj – *Brassica juncea* (acc. no. ABO71662.1-MT1, acc. no. BAB85599.1-MT3), Bo – *Brassica oleracea* (acc. no. AAF70555.1), Ca - *Cicer arietinum* (acc. no. CAA65008.1), Fe - *Fagopyrum esculentum* (acc. no. ABG75912.1), Fr - *Festuca rubra* (acc. no. AAB70464.1), Fxa - *Fragaria x ananassa* (acc. no. P93134.1), Gm – *Glycine max* (acc. no. BAD18376.1-MT1, acc. no. NP_001235506.1-MT2, acc. no. NP_001237328.1-MT4), Ib – *Ipomoea batatas* (acc. no. BAD95645.1), Mxd - *Malus x domestica* (AEJ37039.1), Os – *Oryza sativa* (acc. no. AAC49626.1-MT1, acc. no. NP_001042028.1-MT2, acc. no. NP_001042319.1-MT3, acc. no. NP_001065192.1-MT4), Nc - *Noccaea caerulescens* (acc. no. ACR46965.1), Ps – *Pisum sativum* (acc. no. BAD18382.1), Pxh – *Petunia x hybrida* (acc. no. AAD02561.1), Sm - *Salvia miltiorrhiza* (acc. no. AEQ54919.1), Sn – *Solanum nigrum* (acc. no. ACF10395.1), Sv - *Silene vulgaris* (acc. no. AAC72984.1), Vf– *Vicia faba* (acc. no. CAA62551.1-MT1, acc. no. ACI02063.1-MT4), Zm–*Zea mays* (acc. no. NP_001150528.1-MT1, acc. no. ACG42263.1-MT2, acc. no. NP_001105499.1-MT4).

the arrangement of several Cys residues exist between various plant species. *H. tuberosus* MT2 exhibits one Cys more (15 Cys residues in an 8+7 arrangement, Chang et al., 2004) than rape and other plants. In *Citrus unshiu* Satsuma MT2b, two cysteines are absent from the N-terminal domain (Moriguchi et al., 1998). *Brassica* MT1 exhibit 7 Cys in the second Cys-rich domain, whereas other species harbour one Cys less. In BnMT4 similar to the *A. thaliana* MT4a and 4b (Ren et al., 2012), 17 Cys residues are present.

Analysis of the level of metallothionein transcripts in B. napus organs

Genes encoding metallothioneins are subject to differentiated expression in plant tissues. Many are expressed at high levels. Serial Analysis of Gene Expression (SAGE) has demonstrated that MTs in etiolated rice seedlings comprise 2.7% of all transcripts (Matsumara et al., 1999) and 5% in mature leaves, where *MT3* mRNA is the most abundant (2.8%) (Gibbings et al., 2003). Similarly, a high frequency of MT3 tags was observed in the leaves of the wild banana *Musa acuminata* (Coemans et al., 2005) and during barley malting (White et al., 2006). Numerous MT tags have been observed in potato tubers (Nielsen et al., 2005) and *Arabidopsis* leaves under cold stress (Jung et al., 2003). Additionally, examining ESTs in rice leaves subjected to drought stress revealed that MT genes are expressed at the highest level (Gorantla et al., 2007). The patterns of *BnMT1-4* metallothionein expression in vegetative organs, flowers, pods and *B. napus* seeds were established using the sqRT-PCR method (Fig. 3, 4). The highest level of *BnMT1-3* expression was observed in cotyledons. In other organs of 6-day-old seedlings (roots and hypocotyls) and in the leaves, and pods of 3-month-old plants, *BnMT1* transcript levels were lower or even absent in flowers (Fig. 3). *BnMT4* expression was not demonstrated in the mentioned organs.

A lack of *MT1* expression was also observed in *Arabidopsis* flowers of the same plant family (Zhou and Goldsbrough, 1995). The roots and leaves of 4-week-old *Arabidopsis* plants also lacked *AtMT4a* and *b* transcripts, whereas other MTs exhibit strong organ specificity - *MT1a* was preferentially transcribed in the root, *MT2a* was preferentially transcribed in the leaves, and expression of *MT2b* and *MT3* were stronger in the leaves than the roots (Guo et al., 2003). In whole 7-day-old seedlings of closely related *Brassica rapa*, type 1, 2 and 3 metallothioneins were characterised by almost equal expression levels (Ahn et al., 2012). An experiment with a GUS reporter gene has demonstrated a high *At*M*T* promoters activities in cotyledons from both seedlings and older plants (Guo et al., 2003). In the more evolutionary distant species rice, all 11 MT genes exhibit preferential expression in roots and leaves (Zhou et al., 2006). In hybrid poplar leaves, both *MT* type 2 and *MT3b* gene expression was higher than type 1, in contrast to their expression in roots, and *MT3a* transcripts were indiscernible in both organs (Kohler et al., 2004). Thus, metallothionein expression exhibits species-specific organ specificity, which is also observed for type 2 and 3 *MTs*. *BnMT2* and *3* are expressed in all examined rape organs. *MTs3* expression occurs predominantly in fleshy fruit but can be observed in other organs of species lacking such kinds of fruit, such as leaf mesophyll and roots apices in *A. thaliana* (Guo et al., 2003). *BnMT2* and *3* transcripts in organs of mature rape plants are expressed at steady levels. However, both *M. domestica* and *M. acuminata* exhibit higher *MT3* than *MT2* expression in leaves (Clendennen and May, 1997; Reid and Ross, 1997), in contrast to maize (Freisinger 2008). At least part of the observed differences may result from

Table 2. Primer sequences. The letter g represents genomic.

Primer name	Sequence 5' → 3'	Amplicon	Amplicon size
BnMet1_for	TGGCAGGTTCTAACTGTGGA	*BnMT1* cDNA	309
BnMet1_rev	CAAATGAAAACATTATACACCACACA	*gBnMT1*	444
BnMet2_for	TCAATTTGATTAACATTCTCTGCT	*BnMT2* cDNA	401
BnMet2_rev	AAGCCTGCAGCCATTATTACA	*gBnMT2*	504
BnMet3_for	GCAAAACAACAAAACACACACA	*BnMT3* cDNA	418
BnMet3_rev	CATTACATCACACACCATGC	*gBnMT3*	625
BnMet4_for	GAAGAAAAAGAGCGAGGTAAAA	*BnMT4* cDNA	475
BnMet4_rev	CACCCATTCCCAAGGTATGT	*gBnMT4*	554
Bn5S_for	AGTCGCACAAATCGTGTCTG	*Bn5S rRNA* cDNA	520
Bn5S_rev	TCCATGCTCTCAGCATCAAC		

examining the various species plants at myriad developmental stages. Rape *MT1* and *MT2* gene expression is not limited to one organ, which is observed for *Arabidopsis* (Guo et al., 2003). The presence of *MT2* transcripts in numerous organs has been demonstrated in the phylogenetically distant sweet potato, where expression was apparent in roots, stems and leaves, which harboured the highest expression (Huang et al., 2001). Moreover, numerous *OsMT* genes are expressed in multiple organs (Zhou et al., 2006). A higher *BnMT1-3* gene expression is apparent in young, intensively growing seedling cotyledons as opposed to organs of mature plants. These observations are supported by the results of *in silico* studies (G. Dąbrowska et al., 2012), which reveal that MT promoters of rice and *A. thaliana* harbour a CCGTCC-box element (Silvente et al., 2008) that regulates meristematic cell activation. An accumulation of MT transcripts in intensively proliferating tissues was also observed in other plants (Mir et al., 2004). Reactive oxygen species are a primary signalling factor regulating cell proliferation (Sánchez-Fernández et al., 1997; Vranová et al., 2002; Jiang et al., 2003), suggesting that MTs may maintain a suitable redox state in these tissues (Mir et al., 2004). Because *A. thaliana MT* type 4 genes are expressed exclusively in seeds (Guo et al., 2003), we asked whether *BnMT4* and other cloned rape *MT*s are expressed in dry and germinating seeds. A high level of the *BnMT4* gene transcript was observed in dry seeds, where it was maintained since the 6th hour of imbibition and decreased by almost half in the following 6 hours (Fig 4). Afterwards, *BnMT4* expression decreased only slightly and was at a minimal level in 5-day-old seedlings (Fig 4). The expression pattern for each of the examined *BnMT*s in dry and germinating seeds differed. *BnMT1* and *2* transcripts were detected in all of the examined seed samples. Rape *MT2* transcripts fluctuated the least; however, between the 36th hr and 5th day of germination, a 2-fold difference was apparent. *BnMT1* and *3* genes are expressed differentially during germination. *BnMT1* mRNA was at the highest level at the 6th hr of imbibition - 6-fold higher than that from dry or shortly hydrated seeds. *BnMT3* is expressed after 24 hrs of imbibition and during the subsequent 12 hrs, where it reaches the level at which it is maintained for the next 3.5 days. Metallothionein expression in seeds has been demonstrated in plants other than *Brassicaceae*. Soares et al. (2011) illustrated that non-standardized EST libraries from *Bixa orellana* seeds harbour a relatively high number of sequences with a high similarity to metallothioneins. The first plant MT (E_C protein) was isolated from wheat seeds in a complex with zinc ions. That protein accumulates during a specific developmental stage of a germ, during the transition from proliferation to differentiation, indicating the importance of MT4 for proper development (Kawashima et al., 1992; Robinson et al., 1993). Ren et al. (2012) demonstrated that AtMT4a and AtMT4b *Arabidopsis* proteins are specifically expressed in late embryos, localizing in nuclei, membranes and the cytoplasm. They also confirmed their genes' importance for seed and seedling development by RNAi co-silencing. *MT* type 2 expression in developing root and germ of germinating seeds was observed by Yuan et al. (2008) in rice.

In the *A. thaliana MT1A* gene promoter, the RY motif, a CATGCATG sequence, was identified (Reidt et al., 2000), present in many seed- and maturation-specific gene promoters. It has been suggested that MTs regulate the individual development of a plant perhaps by binding or releasing metal ions that are necessary for proper enzyme and transcription factor function.

Materials and methods

Plant material

We employed the seeds of winter oilseed rape (*Brassica napus* L. var. Kronos, AgroBras Poland) in our experiments. They were sterilized in a mixture of 30% hydrogen peroxide and 96% ethanol in a 1:1 ratio for 5 min and then rinsed with sterile distilled water for 20 min. The seeds were placed in garden soil and cultivated in a culturing chamber at a temperature of 25±1°C with a photoperiod of 16 hrs of light and 8 hrs of darkness. The cotyledons, roots and hypocotyls were collected from 6-day-old seedlings, whereas the leaves, flowers and pods were collected from 3-month-old plants. The organs were frozen in liquid nitrogen and stored at -80ºC until RNA isolation. To examine MT expression levels in in *B. napus* L. seeds, sterile seeds were placed on plates with filter paper moistened with sterile water and left in darkness at 25ºC. After 3, 6, 12, 24, 36, and 48 hrs or 5 days, imbibed and germinating seeds were collected and frozen in liquid nitrogen. Dry, non-germinating seeds were used as a reference sample. The experiment was repeated 3 times.

Identification of rape metallothionein sequences in dbEST

The cDNA sequences of *Arabidopsis thaliana* metallothioneins deposited in NCBI's (National Center for Biotechnology Information) GenBank (NM_100634.1, NM_111773.3, NM_112401.1, NM_129764.1) were used for searching EST *B. napus* sequences. From the numerous ESTs found, the following sequences were selected for further analysis: EV112528.1, GR453238.1, EE479579.1, EE412859.1. Based on these sequences, primer sequences were designed (Table. 2).

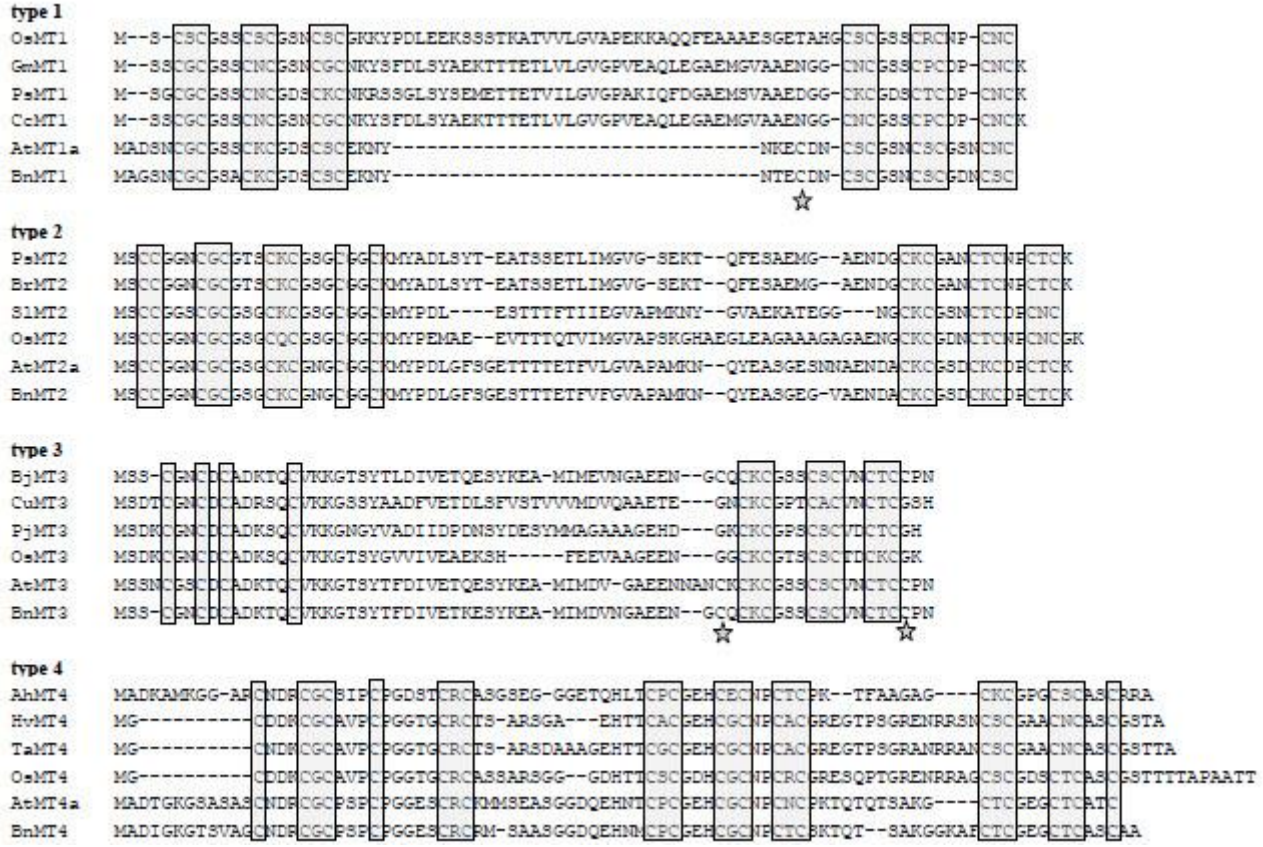

Fig 2. Amino acid sequence of cloned rape *MTs* has cysteine arrangement typical for respective type of plant MTs. Conserved cysteines are boxed. Stars denote additional nonconserved Cys. Ah – *Arachis hypogaea* (GeneBank ABG57067.1), At – *Arabidopsis thaliana* (GeneBank MT1a - NP_172239.1, MT2a - NP_187550.1, MT3 - NP_566509.1, MT4a - NP_179905.1), Bj – *Brassica juncea* (GeneBank BAB85599.1), Bn – *Brassica napus*, Br – *Brassica rapa* (GeneBank BAA11394.1), Cc – *Cajanus cajan* (GeneBank ADD11816.1), Cu – *Citrus unishu* (GeneBank BAF91493.1), Gm – *Glycine max* (GeneBank BAD18376.1), Hv – *Hordeum vulgare* (GeneBank CAD88267.1), Os – *Oryza sativa* (GeneBank MT1 - AAC49626.1, MT2 - NP_001042028.1, MT3 - NP_001042319.1, MT4 - NP_001065192.1), Pj – *Prosopis juliflora* (GeneBank ACC77568.1), Ps – *Pisum sativum* (GeneBank MT1 - BAD18382.1, MT2 - BAD18383.1), Sl – *Solanum lycopersicum* (GeneBank CAA92652.1), Ta – *Triticum aestivum* (GeneBank CAA48351.1).

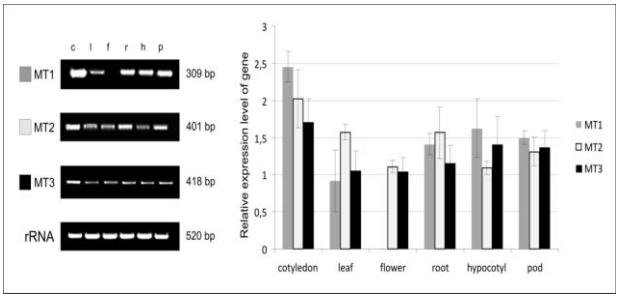

Fig. 3. Comparision of the expression *of BnMT1-BnMT3* in *Brassica napus* organs. Total RNAs were isolated from organs and analyzed by sqRT-PCR using gene-specific primers in three independent experiments. 5S rRNA was amplified to confirm that a constant amount of total RNA was used. The amount of MT mRNA is expressed as the ratio of the densitometric measurement of the sample RT-PCR product to the 5S rRNA corresponding product.

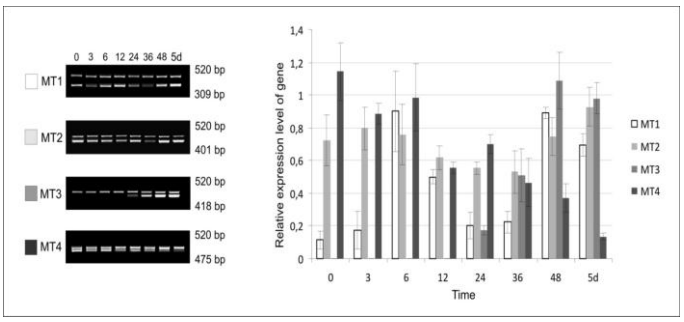

Fig 4. Comparison of the expression of rape *BnMT1-4* in dry (O h) and germinating seeds (3-48 h) and in water-treated 5-day-old seedlings (5 d). Total RNAs were isolated and analyzed by sqRT-PCR using gene-specific primers in three independent experiments. 5S rRNA was amplified to confirm that a constant amount of total RNA was used. The amount of MT mRNA is expressed as the ratio of the densitometric measurement of the sample RT-PCR product to the 5S rRNA corresponding product.

Isolation of nucleic acids and reverse transcription

Genomic DNA and total RNA were isolated from rape organs ground in liquid nitrogen with the Gene MATRIX Plant and Fungi DNA Purification Kit (EURx) and TRI Reagent, (Sigma) respectively. One microgram of total RNA was treated with 200 U DNaseI and incubated at 37°C for 30 min to remove any DNA contamination. Next, the enzyme was inactivated by addition of 0.025 mM EDTA at 65ºC for 10 min. Next, the entire extract was used for reverse transcription reactions with MMLV-RT (Novazyme).

Cloning and sequencing rape MT

The cDNA obtained from reverse transcription and the genomic DNA were used as templates for PCR with starters specific for particular MT sequences (Table. 2). The amplification reaction with the cDNA template was conducted as follows: 94°C 2 min, (94°C 30 s, 53-57°C 30 s, 7 °C 45 s) x 30, 72°C 7 min. The above conditions were used for PCR with the genomic DNA template but with an annealing temperature of 51-53°C. PCR products were separated in a 1% agarose gel, eluted from the gel using Gene MATRIX Agarose-Out DNA Purification Kit (EURx), and ligated to the pJET1.2 vector (Fermentas) according to the Clone JET PCR Cloning Kit manual (Fermentas). The ligation mixture was used to transform *E. coli* XL1blue bacteria using the heat shock method (Sambrook et al., 1989). Plasmid DNA was isolated using the Gene MATRIX Plasmid Miniprep DNA Purification Kit (EURx). The sequencing of cloned sequences was performed in the Laboratory of DNA Sequencing and Oligonucleotides Synthesis of Institute of Biochemistry and Biophysics, Polish Academy of Science in Warsaw.

Semi-quantitative RT-PCR (sqRT-PCR)

The cDNA obtained from particular organs was used as a template in sqRT-PCR reactions. For each organ, reactions with primer pairs for four metallothionein genes (*BnMT1-BnMT4)* were performed. The reaction with primers for *BnMT4* was performed with cDNA from dry and germinating *B. napus* seeds. PCR reactions were optimized for distinct genes. For *BnMT1*, *BnMT3* and *Bn5S rRNA* genes, 26 PCR cycles were performed, whereas 28 cycles were performed for *BnMT2* and *BnMT4*. The reaction mixture contained 1/20 of RT reaction volume, 0.2 μM of each primer, 0.6 U of Taq polymerase (Promega) and other components dictated by the protocol. The amplification extraction was conducted using the following conditions: 2 min for 95°C (30 s for 95°C, 30 s for 53°C, 40 s for 72°C) x 26-28, 5 min at 72°C. PCR products were separated in 2% or 2.5% agarose gels in TAE buffer (Tris-Acetate-EDTA) for 1 hr at 90 V. After electrophoresis, the gels were visualized under UV, and their images were used to quantify the amount of PCR product by densitometric examination. ImageGauge 3.46. software was used for signal quantification. Each reaction was repeated three times, and the error bars represent the standard deviation of the mean.

In silico analysis of B. napus MT

Rape metallothionein sequences were analyzed using BLAST software(http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Phylogenetic analysis of plant MT sequences was conducted using the MEGA 4.1 Neighbor-Joining algorithm (Tamura et al., 2007). Evolutionary distances were computed using the Poisson correction method, presented using the units of the number of amino acid substitutions per site. A bootstrap test with 1,000 replicates was applied to assess the reliability of the phylogenetic tree.

Conclusions

Taken together, our results demonstrate that *BnMT* genes of various types are transcribed in an organ-specific manner. The level of *BnMT1* and *2* metallothioneins in rape organs can be arranged in the following descending order of expression: cotyledon > root > hypocotyl > leaf > flower. The presence of numerous metallothionein isoforms in plants and their distinct models of expression suggest numerous roles for MTs in plant cells. Our analyses of MT promoter sequences in *A. thaliana* and *O. sativa* conducted *in silico* demonstrated the presence of *cis*-elements responsible for specific MT expression in roots and seeds, which may additionally confirm organ-specific plant MT expression, including rape metallothioneins (Dąbrowska et al., 2012).
MTs are known as metal chelators and putative ROS scavengers. A high level of MT gene expression in different organs demonstrates an important role for these genes during

the development of plants from seed germination to flowering. Because proper plant development is one of the main characteristics that heavily influences the productivity of crops, expanding our knowledge about MTs function may conduce to promoting of plant fecundity.

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