Genome-Wide Characterization and Analysis of Metallothionein Family Genes That Function in Metal Stress Tolerance in *Brassica napus* L.

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**Abstract:** *Brassica* plants exhibit both high biomass productivity and high rates of heavy metal absorption. Metallothionein (MT) proteins are low molecular weight, cysteine-rich, metal-binding proteins that play crucial roles in protecting plants from heavy metal toxicity. However, to date, MT proteins have not been systematically characterized in *Brassica*. In this study, we identified 60 MTs from *Arabidopsis thaliana* and five *Brassica* species. All the MT family genes from Brassica are closely related to *Arabidopsis* MTs, encoding putative proteins that share similar functions within the same clades. Genome mapping analysis revealed high levels of synteny throughout the genome due to whole genome duplication and segmental duplication events. We analyzed the expression levels of 16 *Brassica napus* MTs (BnaMTs) by RNA-sequencing and real-time RT-PCR (RT-qPCR) analysis in plants under As$^{3+}$ stress. These genes exhibited different expression patterns in various tissues. Our results suggest that BnaMT3C plays a key role in the response to As$^{3+}$ stress in *B. napus*. This study provides insight into the phylogeny, origin, and evolution of MT family members in *Brassica*, laying the foundation for further studies of the roles of MT proteins in these important crops.

**Keywords:** metallothionein; *Brassica*; *Brassica napus*; As$^{3+}$ stress

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**1. Introduction**

Heavy metals are essential micronutrients for various physiological processes in plants. However, excess amounts of essential (copper, zinc) and non-essential metals (cadmium) are toxic to plants, as they inhibit plant growth, impair root development, and decrease chlorophyll contents, resulting in chlorosis [1,2]. Therefore, plants have evolved a suite of mechanisms involving the chelation and sequestration of heavy metals by various amino acids, organic acids, phytochelatins (PCs) and metallothioneins (MTs) [3,4]. These compounds play crucial roles in protecting plants from heavy metal toxicity [5–9].
MTs, low-molecular-weight proteins (7–10 kDa) with a high percentage of cysteine (Cys) residues [10,11], have been widely characterized in various prokaryotic and eukaryotic organisms. Plant MTs are classified into four types according to the arrangement of their Cys residues [12], including the MT1, MT2, MT3, and MT4 subfamilies [10,13]. MTs play crucial roles in ion homeostasis and tolerance in plants. Seven functional MT genes have been isolated from the model plant Arabidopsis thaliana. Of these, AtMT1a, AtMT2a, AtMT2b, and AtMT3 enhance plant tolerance of Cu ions, especially in leaves [14,15], as well as Cd tolerance in transformed yeast and fava bean (Vicia faba) guard cells [16,17]. AtMT4 modulates Zn homeostasis in seeds and is highly expressed during the late stages of development [18]. Additionally, various MT genes show significantly different expression patterns in plants under heavy metal stress. For example, MT2a and MT2b genes are more highly expressed in the roots of the heavy metal hyperaccumulator Noccaea caerulescens than in those of A. thaliana, while MT3 is more highly expressed in shoots of N. caerulescens than in the non-hyperaccumulator Thlaspi arvense [19,20]. MT4 mRNAs are primarily expressed in ripening fruits and developing seeds [14]. Therefore, plant MTs are likely involved in many physiological processes, such as seed development and germination [18,21,22], fruit ripening [14], and root development [21,23]. 

Brassica plants are considered to be highly tolerant to heavy metals (e.g., Cd, Cu, Ni, Zn, Pb, and Se), making them ideal plants for studying metal accumulation in phytoremediation studies [24–27]. Indian mustard (Brassica juncea) is a high-biomass-producing crop with the potential to take up and accumulate heavy metals [1,23,27,28]. However, this plant accumulates Cd less effectively than other crops such as maize (Zea mays), rice (Oryza sativa), and sugar beet (B. vulgaris) when it is present at low concentrations in the soil [29]. Rapeseed (B. napus) has many advantages for this type of analysis due to its rapid growth, high biomass productivity and efficient heavy metal absorption, and it is therefore also widely used to investigate heavy metal tolerance [26,30–32]. Indeed, while heavy metal tolerance has been well studied in various Brassica species, the mechanisms that contribute to the tolerance of these plants to heavy metals remain unclear.

In the present study, we screened the draft genome sequences of A. thaliana and various Brassica species (Brassica rapa, Brassica oleracea, B. napus, Brassica juncea and Brassica nigra) for MT genes that participate in heavy metal detoxification. We identified 60 MT genes and performed a detailed analysis of their duplication patterns, classifications, and chromosomal distribution and motifs, as well as a phylogenetic analysis. Finally, we verified the differential expression profiles of selected rapeseed MT genes in different B. napus tissues at various developmental stages. We also investigated the expression patterns of MT genes in B. napus seedlings exposed to heavy metals. Our results provide important information about the origin and evolution of the MT gene family in Brassica and provide a basis for further studies of the functions of MT family proteins in rapeseed.

2. Results

2.1. Identification and Multiple Sequence Alignment of MT Family Genes

Using the protein sequences of the MT family genes from the TAIR10 database (Table 1) as queries, we identified 60 MT genes in A. thaliana and various Brassica plants (B. rapa, B. oleracea, B. napus, B. juncea and B. nigra). These genes were classified into four subgroups (Figure 1, Table 1). Of these, seven were MT1 subfamily members, five of which were identified from the corresponding genome databases, except BolMT1 and BjuMT1. BraMT1 has been reported in B. rapa with 45 amino-acid proteins [33]; the difference between these sequences requires further study. In addition, 37 were MT2 subfamily members, encoding deduced proteins ranging from 56 to 103 amino acids in length; nine were MT3 subfamily members, encoding proteins from 65 to 69 amino acids in length; and seven were MT4 subfamily members, encoding proteins from 85 to 120 amino acids in length (Table 1). Of the MT1 subfamily members, three homologs were identified in A. thaliana, while BnaMT1 and BraMT1 were identified in B. napus and B. rapa, respectively. No MT1 subfamily members were found in B. oleracea, B. juncea, or B. nigra, whereas BolMT1 and BjuMT1 have been reported previously [13].
indicating that genome gaps may still emerge in *B. oleracea*, and *B. juncea*. Six Cys-X-Cys motifs were equally distributed on the N- and C-termini of MT1 family proteins, except in the case of BraMT1 and AtMT1B (Figure 2A).

**Figure 1.** Neighbor-Joining (NJ) phylogenetic tree showed the relationships of Metallothionein (MT) family genes from *A. thaliana* and various *Brassica* species. The rooted neighbor-joining phylogenetic tree was constructed using MEGA6 and visualized using Figure Tree v1.4.2. The MTs were divided into four subfamilies (MT1–MT4), which are indicated by different colors. Organism name and gene accession numbers are shown in Table 1.

We identified 37 MT2 subfamily genes in *A. thaliana* (AtMT2A and AtMT2B) and *Brassica* (five in *B. rapa*, six in *B. oleracea*, ten in *B. napus*, nine in *B. juncea*, and five in *B. nigra*), which were divided into the MT2A and MT2B subgroups (Figure 2B, Table 1), pointing to the extensive triplication and expansion of these genomes during their evolution in *Brassica* plants. Furthermore, one Cys–Cys and two Cys–X–Cys motifs were almost always present in the N-terminal regions of these proteins, and three Cys–X–Cys were almost always present in their C-terminal regions (Figure 2B). In addition, MT2 subfamily genes encode a deduced protein with the MSCCGGN/S sequence in their N-termini, which is consistent with previous findings [13,34]. Three variant regions were found in the MT2A subgroup and three in the MT2B subgroup (Figure 2B), which might be associated with their roles in metal tolerance.

We identified MT3 subfamily genes, including one each in *A. thaliana*, *B. rapa*, *B. juncea*, and *B. nigra*, two in *B. oleracea* and three in *B. napus* (Figure 1, Table 1). The alignment of the MT3 amino acid sequences showed a completely conserved sequence, CXXCDCX3C, located in the N-terminus of each protein, and a highly conserved consensus sequence with eight Cys residues at the C-terminus (Figure 2C). In addition, 30–40 amino acids were detected in the Cys-poor linker region between the N- and C-terminal regions, in accordance with the MT2 subfamily (Figure 2B,C), pointing to a possible evolutionary relationship between the MT2 and MT3 family genes.
Table 1. List of Metallothionein (MT) genes identified in the *A. thaliana* and *Brassica* genomes.

| Groups | Name       | Gene ID       | Chr.  | Start (bp) | End (bp) | Length (bp) | Length (aa) | MW (KDa) | pIs | Exon | Intron |
|--------|------------|---------------|-------|------------|----------|-------------|-------------|----------|-----|------|--------|
| MT1    | AtMT1      | AT1G07600     | AtChr1| 2338904    | 2339321  | 138         | 45          | 4.580   | 4.23 | 2    | 1      |
| MT1    | AtMT1C     | AT1G07610     | AtChr1| 2341542    | 2342123  | 138         | 45          | 4.495   | 4.54 | 2    | 1      |
| MT1    | AtMT1B     | AT5G56795     | AtChr5| 22972042   | 22972449 | 156         | 51          | 5.428   | 10.25| 2    | 1      |
| MT1    | BraMT1     | Bra015594     | BraA10| 766670     | 772706   | 450         | 149         | 16.77   | 9.32 | 4    | 3      |
| MT1    | BnaMT1     | BnaA10g04950D | BnaA10| 2673266    | 2673770  | 138         | 45          | 4.480   | 3.92 | 2    | 1      |
| MT1    | BolMT1     | DK501359      | UN    | 138        | 45        | 4.439       | 3.92        | UN      | UN   | UN   | UN     |
| MT1    | BjuMT1     | EF471214      | UN    | 138        | 45        | 4.439       | 3.92        | UN      | UN   | UN   | UN     |
|        | AtMT2A     | AT3G09390     | AtChr3| 288946     | 2890229  | 246         | 81          | 8.163   | 4.35 | 2    | 1      |
|        | AtMT2B     | AT5G02380     | AtChr5| 506498     | 507244   | 234         | 77          | 7.766   | 4.54 | 2    | 1      |
|        | BraMT2A    | Bra001309     | BraA03| 15803598   | 15803933 | 246         | 81          | 8.197   | 4.17 | 2    | 1      |
|        | BraMT2B    | Bra005720     | BraA03| 275453     | 275766   | 243         | 80          | 8.033   | 4.29 | 2    | 1      |
|        | BraMT2C    | Bra009595     | BraA10| 16182058   | 16182453 | 243         | 80          | 8.031   | 4.29 | 2    | 1      |
|        | BraMT2D    | Bra028875     | BraA02| 269835     | 270238   | 246         | 81          | 8.386   | 4.20 | 2    | 1      |
|        | BraMT2E    | Bra029765     | BraA05| 23082833   | 23083178 | 243         | 80          | 8.024   | 4.35 | 2    | 1      |
|        | BolMT2A    | Bol000591     | Scaffold000521 | 37950   | 38342   | 243         | 80          | 8.031   | 4.29 | 2    | 1      |
|        | BolMT2B    | Bol011307     | Scaffold000212 | 445183 | 445551 | 243         | 80          | 8.054   | 4.58 | 2    | 1      |
|        | BolMT2C    | Bol012825     | BolC02 | 305134     | 305536   | 246         | 81          | 8.386   | 4.20 | 2    | 1      |
|        | BolMT2D    | Bol015273     | BolC03 | 101213     | 101535   | 243         | 80          | 8.137   | 4.29 | 2    | 1      |
|        | BolMT2E    | Bol023080     | BolC01 | 37533909   | 37534079 | 171         | 56          | 5.920   | 4.15 | 1    | 0      |
|        | BolMT2F    | Bol033924     | Scaffold00040 | 316614 | 316949 | 246         | 81          | 8.147   | 4.15 | 2    | 1      |
|        | BnaMT2A    | BnaA03g30680D | BnaA03| 14857530   | 14858170 | 240         | 79          | 7.966   | 3.81 | 2    | 1      |
|        | BnaMT2B    | BnaA03g54880D | A03_random | 44751   | 45330   | 243         | 80          | 8.077   | 4.29 | 2    | 1      |
|        | BnaMT2C    | BnaA05g29011D | BnaA05 | 20416060  | 20416808 | 243         | 80          | 8.024   | 4.35 | 2    | 1      |
|        | BnaMT2D    | BnaA10g27170D | BnaA10 | 17170773  | 17171571 | 243         | 80          | 8.031   | 4.29 | 2    | 1      |
|        | BnaMT2E    | BnaAnng00330D | Bna_A03 | 321245   | 322046   | 246         | 81          | 8.370   | 4.20 | 2    | 1      |
| MT2    | BnaMT2F    | BnaC02g03550D | BnaC02 | 1685618   | 1686330  | 246         | 81          | 8.386   | 4.20 | 2    | 1      |
| MT2    | BnaMT2G    | BnaC03g00710D | BnaC03 | 346945    | 347282   | 246         | 71          | 7.284   | 4.08 | 2    | 1      |
| MT2    | BnaMT2H    | BnaC03g39600D | BnaC03 | 21778025  | 21778470 | 240         | 79          | 7.950   | 3.79 | 2    | 1      |
| MT2    | BnaMT2I    | BnaC05g34390D | BnaC05 | 40327486  | 40328304 | 243         | 80          | 8.028   | 4.58 | 2    | 1      |
| MT2    | BnaMT2J    | BnaCnnng04900D | Cnn_random | 38972401  | 38973067 | 243         | 80          | 8.031   | 4.29 | 2    | 1      |
|        | BjuMT2A    | BjuA008858    | BjuA03 | 321697    | 322020   | 243         | 80          | 8.033   | 4.29 | 2    | 1      |
|        | BjuMT2B    | BjuA020647    | BjuA05 | 29317680  | 29319366 | 321         | 103         | 10.661  | 4.24 | 3    | 2      |
|        | BjuMT2C    | BjuA040818    | BjuA02 | 430697    | 431097   | 246         | 81          | 8.370   | 4.20 | 2    | 1      |
|        | BjuMT2D    | BjuA044587    | BjuA10 | 19619066  | 19619458 | 243         | 80          | 8.031   | 4.29 | 2    | 1      |
|        | BjuMT2E    | BjuB001621    | BjuB08 | 169755    | 170152   | 243         | 80          | 8.059   | 4.29 | 2    | 1      |
| Groups | Name            | Gene ID       | Chr.          | Start (bp) | End (bp)  | Length (bp) | Length (aa) | MW (KDa)  | pIs  | Exon | Intron |
|--------|-----------------|---------------|---------------|------------|-----------|-------------|-------------|-----------|------|------|--------|
|        | BjuMT2F         | BjuB005939    | BjuB01        | 43381550   | 43383641  | 297         | 98          | 10.057    | 3.93 | 3    | 2      |
|        | BjuMT2G         | BjuB012072    | BjuB05        | 20387272   | 20387699  | 246         | 81          | 8.353     | 4.11 | 2    | 1      |
|        | BjuMT2H         | BjuB031838    | BjuB03        | 12766757   | 12767103  | 222         | 73          | 7.596     | 4.35 | 2    | 1      |
|        | BjuMT2I         | BjuB044439    | BjuB02        | 52757313   | 52757655  | 243         | 80          | 8.061     | 4.29 | 2    | 1      |
|        | BniMT2A         | BniB001954-PA | BniB08        | 30524166   | 30524483  | 243         | 80          | 8.075     | 4.29 | 2    | 1      |
|        | BniMT2B         | BniB007929-PA | BniB05        | 2189985    | 2190357   | 243         | 80          | 7.978     | 4.35 | 2    | 1      |
|        | BniMT2C         | BniB023579-PA | BniB02        | 28213025   | 28213453  | 246         | 81          | 8.353     | 4.11 | 2    | 1      |
|        | BniMT2D         | BniB039464-PA | BniB07        | 32240383   | 32240733  | 222         | 73          | 7.596     | 4.35 | 2    | 1      |
|        | BniMT2E         | BniB045064-PA | BniB03        | 44318189   | 44318591  | 225         | 74          | 7.569     | 4.08 | 2    | 1      |
| MT3    | AtMT3           | AT3G15353     | AtChr3        | 5180642    | 5181586   | 210         | 69          | 7.373     | 4.35 | 3    | 2      |
|        | BraMT3          | Bra027254     | BraA05        | 20683494   | 20683920  | 204         | 67          | 7.183     | 4.17 | 3    | 2      |
|        | BolMT3A         | Bol011145     | BolC05        | 28120530   | 28120940  | 204         | 67          | 7.158     | 4.15 | 3    | 2      |
|        | BolMT3B         | Bol025753     | BolC03        | 17061982   | 17062438  | 198         | 63          | 7.016     | 4.40 | 3    | 2      |
|        | BnaMT3A         | BnaA05g24200D | BnaA05        | 18177871   | 18178669  | 204         | 67          | 7.127     | 4.15 | 3    | 2      |
|        | BnaMT3B         | BnaC03g39060D | BnaC03        | 24091592   | 24092154  | 198         | 65          | 7.016     | 4.40 | 3    | 2      |
|        | BnaMT3C         | BnaC05g38240D | BnaC05        | 37017131   | 37017881  | 204         | 67          | 7.158     | 4.15 | 3    | 2      |
|        | BjuMT3          | BjuB025665    | BjuB01        | 40434253   | 40434691  | 204         | 67          | 7.187     | 4.15 | 3    | 2      |
|        | BniMT3          | BniB08959-PA  | BniB05        | 4957466    | 4957899   | 204         | 67          | 7.187     | 4.15 | 3    | 2      |

| MT4    | AtMT4B          | AT2G23240     | AtChr2        | 9898585    | 98986325  | 261         | 86          | 8.437     | 5.58 | 2    | 1      |
|        | AtMT4A          | AT2G42000     | AtChr2        | 17529243   | 17530443  | 366         | 121         | 12.229    | 7.62 | 4    | 3      |
|        | BraMT4          | Bra000590     | BraA03        | 11951235   | 11951571  | 261         | 86          | 8.480     | 7.37 | 2    | 1      |
|        | BnaMT4A         | BnaA05g23200D | BnaA03        | 11067719   | 11068213  | 261         | 86          | 8.480     | 7.37 | 2    | 1      |
|        | BnaMT4B         | BnaC03g27400D | BnaC03        | 15895032   | 15895647  | 261         | 86          | 8.468     | 6.97 | 2    | 1      |
|        | BjuMT4          | BjuO006263    | Contig407_1_341981 | 122112  | 122451   | 261         | 86          | 8.500     | 6.97 | 2    | 1      |
|        | BniMT4          | BniB049568-PA | BniB03        | 29749524   | 29749863  | 261         | 86          | 8.472     | 6.97 | 2    | 1      |

Note, At, A. thaliana; Bra, B. rapa; Bol, B. oleracea; Bni, B. nigra; Bna, B. napus; and Bju, B. juncea; Chr., Chromosome; UN, unknown.
Based on the multiple sequence alignment of the deduced MT1–MT4 proteins, it was found that Cys-rich regions are widely distributed among MT family proteins. These regions are characterized by conserved consensus sequences, with motifs such as Cys–G–Cys, Cys–K–Cys, and Cys–S–Cys (Figure 2). To investigate the evolutionary relationships among MT family genes from *A. thaliana* and various *Brassica* species, we constructed a NJ phylogenetic tree based on the alignment of MT domains. Based on the phylogenetic tree, the 58 MT domains were classified into four subfamilies (*MT1*, *MT2*, *MT3*, and *MT4*), except for *BolMT1* and *BjuMT1*, which were not annotated in the genome databases, and most genes were grouped with the *AtMTs* (Figure 2). *AtMT1B* represents the outgroup in the phylogenetic tree. In addition, the *MT2* subfamily was classified into two sister groups (Figure 1), *MT2A* and *MT2B*, which is highly consistent with the results of multiple sequence alignments of whole proteins (Figure 2). For example, two sister groups were also identified and found to contain eight and six *MT* family genes, respectively (Figure 2), which also contain highly conserved consensus...
sequences (Figure 1). These results will be helpful in identifying the functions of MT family genes via orthology analysis.

2.3. Genomic Structure and Conserved Motif Analysis of the MT Gene Family

We characterized the gene structures of the MT family genes by comparing the full-length CDS and the corresponding genomic DNA sequences using GSDS 2.0 (http://gsds.cbi.pku.edu.cn/index.php). Of the 58 MT genes, 44 contain a single intron with a highly conserved structure in each group, i.e., the MT1, MT2 and MT4 subfamilies. Additionally, MT3 subfamily members contain two introns, which were also found in BjuMT2B and BjuMT2F (Figure 3). BolMT2E lacks an intron and belongs to the MT2A gene family, while AtMT4A and BraMT1 contain three introns with distinct sizes (Figure 3). Most genes in the same subfamily exhibit similar exon–intron structures, but the genomic structures of BjuMT2B and BjuMT2F are similar to those of the MT3 subfamily, providing further support for the evolutionary relationship and classification of the MT gene family members identified in this study.

![Figure 3](image_url)

**Figure 3.** Phylogenetic relationships and genomic structures of the MT genes from *A. thaliana* and various *Brassica* species. The red boxes represent exons, solid lines represent introns (connecting two exons), and blue boxes represent untranslated regions (UTRs). The lengths of the MT genes are indicated by horizontal lines (kb).

Using MEME v4.12.0 (http://meme-suite.org/tools/meme), six, eight, three, and four conserved motifs were detected in the MT1, MT2, MT3, and MT4 subfamilies, respectively, in *B. rapa*, *B. oleracea*, *B. napus*, *B. juncea*, and *B. nigra* (Figure 4A–D); the detailed structures of the motifs are shown in Figure S1A–D. All members of the MT1 subfamily except for AtMT1B contain motif 1 (Figure 4A and Figure S1A). All members of the MT2 subfamily contain motif 1, whereas all members of the MT2A subfamily contain motifs 2, 4, and 6, but motif 3 is found only in the MT2B subfamily members. The motifs in the MT2B subfamily members are more variable than those of the other MTs (Figure 4B and Figure S1B), pointing to the triplication and expansion of *Brassica* genomes. All nine genes in
the MT3 subfamily contain motif 1, while the MT3A subfamily genes contain motif 2 and the MT3B subfamily genes contain motif 3 (Figure 4C and Figure S1C). The MT4 subfamily genes contain motifs 1, 2, and 3, indicating that these motifs are conserved among these genes (Figure 4D and Figure S1D). In summary, the same conserved motifs are widely found in paralogous/orthologous genes, suggesting that they might have similar functions at the protein level.

Figure 4. Putative conserved motifs in MT family proteins in various Brassica species identified using the MEME search tool. (A) the conserved motifs in MT1 family; (B) the conserved motifs in MT2 family; (C) the conserved motifs in MT3 family; (D) the conserved motifs in MT4 family. Different motifs are represented by different colors, and protein names and combined p values are shown on left side of this figure. The best possible matched motifs, their functional annotation, and motif width are shown in Figure S1.
2.4. Chromosome Locations and Duplication of MT Genes in Brassica

Brassica includes three diploid species, B. rapa (AA, 2n = 20), B. oleracea (CC, 2n = 18), and B. nigra (BB, 2n = 20) and three allotetraploid species, B. napus (AACC, 2n = 38), B. juncea (AABB, 2n = 36), and B. carinata (BBCC, 2n = 34), and the evolution and relationships between the members of Brassica can be well understood according to the U-triangle theory [35]. Five of these species have been completely sequenced, and their sequences are available in the Brassica database (BRAD) database. To identify the physical positions of the MT genes, we mapped them to the chromosomes in the corresponding Brassica species. The 43 MT genes are located on 27 chromosomes in the five Brassica species with available whole-genome sequences, including four chromosomes (BraA02, BraA03, BraA05, and BraA10) in B. rapa, four chromosomes (BolC01, BolC02, BolC03 and BolC05) in B. oleracea and five chromosomes in B. nigra (BniB02, BniB03, BniB05, BniB07 and BniB08) (Figure 5).

Further, we detected high levels of synteny among MT family genes in these species. For example BraMT2D on chromosome BraA02, BjuMT2C on chromosome BjuA02, BnaMT2F on chromosome BnaC02, and BolMT2C on chromosome BolC02 are located near the top of the chromosomes and are classified into the same subgroups (Figure 2), suggesting that these genes might have undergone whole-genome duplication events during the evolutionary process and might have similar functions. However, some of these genes, e.g., BnaMT4A and BraMT4 on chromosome A03, BnaMT3A and BraMT3 on chromosome A05, and BnaMT1 and BraMT1 on chromosome A10 might have undergone segmental duplications (Figure 5). Finally, BjuMT2H and BniMT2E on chromosome B03 and BjuMT2E and BniMT2A on chromosome B08 might have undergone gene transposition (Figure 5). Taken together, these results shed light on the evolutionary patterns of these subfamilies among adjacent species.

![Figure 5. Chromosomal distribution and analysis of duplication events in MT family genes among Brassica species. Genes from the same subgroups are indicated by the same color, which is consistent with the corresponding family in the phylogenetic tree (Figure 1). The labels on the corresponding chromosomes indicate the names of the source organism and the subgenome. The scales indicate the sizes of various Brassica plant genomes (Mb). Bra, B. rapa; Bol, B. oleracea; Bni, B. nigra; Bna, B. napus; and Bju, B. juncea. The genes located on the scaffold are not shown in the Figure 5.](image-url)
2.5. Expression Profiles of BnaMT Family Genes in B. napus

Based on the transcriptome sequencing datasets from *B. napus* ZS11 (BioProject ID PRJNA358784), we characterized the expression profiles of the *BnaMT* genes in eight different tissues, covering all stages of rapeseed development (Figure 6, Tables S1 and S2). Among the 16 *BnaMT* genes, *BnaMT1* was more highly expressed in the stems, leaves, and siliques 30 days after pollination than in other tissues (Figure 6). Among MT2 genes, *BnaMT2A* and *BnaMT2H* were more highly expressed in the stems, leaves, and siliques 30 days after pollination than in other tissues (Figure 6). Among MT2 genes, *BnaMT2A* and *BnaMT2H* were specifically expressed in buds; *BnaMT2C* and *BnaMT2I* were more highly expressed in stems, leaves, and siliques before day 30 than in other tissues (Figure 6). *BnaMT3A*, *BnaMT3B*, and *BnaMT3C* were more highly expressed in stems, leaves, and siliques before day 30 than in other tissues (Figure 6). Finally, *BnaMT4A* and *BnaMT4B* were more highly expressed in stems, leaves, and siliques before day 30 than in other tissues (Figure 6).

![Figure 6. Heatmap of the expression profiles of BnaMT family genes in different tissues and organs.](http://hemi.biocuckoo.org/contact.php)

The abbreviations above the heatmap indicate the different tissues and organs/developmental stages of *B. napus* ZS11 (listed in Table S1). The expression data was gained from the RNA-seq data and shown as log2, as calculated by fragments per kilo base of exon model permillion (FPKM) values. Black boxes indicate that no expression was detected by RNA-seq analysis. The heatmap was generated using Heatmap Illustrator v1.0 (Hemi v1.0, Huazhong University, Wuhan, China; http://hemi.biocuckoo.org/contact.php).

2.6. Expression Analysis of BnaMT Genes in Response to Metal Treatment

MTs are the best-characterized heavy-metal-binding ligands in plants. To analyze the roles of *BnaMTs* in metal tolerance, we compared the expression profiles of *BnaMTs* in the roots, hypocotyls, and cotyledons of *B. napus* plants under As$^{3+}$ stress versus normal conditions via real-time RT-PCR.
(RT-qPCR). Under normal conditions, the expression patterns of the *BnaMTs* were similar to the patterns identified by RNA-seq, with different expression profiles detected among different rapeseed varieties (Figure 7, Table S3). For example, *BnaMT2B, BnaMT2C, BnaMT2D*, and *BnaMT2J* were highly expressed in all tissues; *BnaMT1* and *BnaMT4B* were expressed at lower levels in roots, hypocotyls, and cotyledons; *BnaMT2A, BnaMT2F, BnaMT2G, BnaMT2H, BnaMT2J, BnaMT3A*, and *BnaMT3B* were expressed at lower levels in roots and hypocotyls than in cotyledons (Figure 7); and *BnaMT2A* and *BnaMT2H* did not exhibit tissue-specific expression in *B. napus* (Figures 6 and 7). After As$^{3+}$ treatment, all *BnaMT* genes were expressed at higher levels in roots than in hypocotyls but were expressed at the highest levels in cotyledons (Figure 7). For example, *BnaMT1* was upregulated by As$^{3+}$ treatment, and *BnaMT2A, BnaMT2B, BnaMT2F, BnaMT2J, and BnaMT3B* were more significantly upregulated in cotyledons than in roots and hypocotyls (Figure 7). Importantly, *BnaMT3C* was more highly expressed in varieties B33 and B34 than in B93 and B113 (Figure 7).

Figure 7. Expression analysis of *BnaMT* family genes in different tissues under control and As$^{3+}$ treatment via real-time RT-PCR (RT-qPCR). Three biological replicates per sample were used for analysis, and three technical replicates were analyzed per biological replicate. Values represent the average of three biological replicates with three technical replicates of each tissue (Table S3). The expression data was gained from the real-time RT-PCR (RT-qPCR) analysis data and shown as log2 as calculated by average values normalized to that of the reference gene *BnACTIN7* (EV116054). *, ** indicates a significance level at 0.05 and 0.01, respectively. The heatmap was generated using Heatmap Illustrator v1.0 (Hemi v1.0, Huazhong University, Wuhan, China; http://hemi.biocuckoo.org/contact.php).

3. Discussion

The high-affinity heavy metal chelators, PCs and MTs, play crucial roles in maintaining metal homeostasis during plant development [36–41]. Moreover, the *Brassica* plants had high biomass...
productivity and high levels of heavy metal absorption, as analyzed in *B. juncea* [1,23], *B. rapa* [33], and *B. napus* [42]. In addition, seven putative MT genes have been identified in *Arabidopsis* [15,16], but no comprehensive study of these genes has been reported. *Brassica* species, which were derived from a common ancestor, are ideal model systems for analyzing polyploid evolution and genome duplication [43]. Many analyses have focused on the model plant *A. thaliana* and various *Brassica* species (*B. rapa*, *B. nigra*, *B. oleracea*, *B. napus*, *B. juncea* and *B. carinata*). The whole genome sequences of all species except *B. carinata* are available in BRAD (the *Brassica* Database, http://brassicadb.org/brad/downloadOverview.php). In the present study, we identified 52 MT genes from various *Brassica* species based on *A. thaliana* MT gene sequences (Table 1). Phylogenetic analysis revealed that all MT family genes are closely associated with *AtMTs* (Figure 2), suggesting that they share similar functions or have undergone gene fusion [44]. Of these, *MT1* subfamily genes from *B. nigra*, *B. oleracea*, and *B. juncea* have not been identified, but *BoMT1* and *BjuMT1* have been identified [13]. The number of *MT2* (10) and *MT3* (3) genes in *B. napus* is nearly equal to the sum of these genes in *B. rapa* (5 MT2 and 1 MT3) and *B. oleracea* (6 MT2 and 2 MT3), and most genes showed high levels of synteny throughout the genome, reflecting the fact that whole genome duplications and segmental duplications were a major contributor to the expansion of *MTs* during evolution. However, the deduced protein sequence of *BraMT1* in *B. rapa* is longer than the previously published sequence [33], indicating the need for further study to confirm *BraMT1*. In addition, homologs of *BnaMT4A* and *BraMT4*, *BnaMT3A* and *BraMT3*, and *BnaMT1* and *BraMT1* were not detected in the corresponding genomes (Figure 5, Table 1), in accordance with the finding that gene loss typically occurs after polyploidization in eukaryotes [45–47]. Furthermore, Cys-rich regions were almost completely conserved among MTs, and the distinct spacer sequences in the Cys-poor linkers were also well-conserved, comprising 7 amino acids in *Brassica* MT1s, 40–42 amino acids in *Brassica* MT2s, 32–34 amino acids in *Brassica* MT3s, and 14–15 amino acids in *Brassica* MT4s (Figure 1). These results are in close agreement with previous predictions that Cys-rich regions will show highly conserved MT family genes [13,33], and we infer that the variations in the MTs might be associated with their different functions in plants [48].

To date, plant MTs have been widely characterized, exhibiting different tissue-specific expression patterns [8,14,49–52]. For example, *AtMT1A* and *AtMT2B* were predominantly expressed in roots and leaves, while *AtMT2A* and *AtMT3* were highly expressed in roots and young leaves [14]. Likewise, notable differences in expression patterns were also found among *MT1*, *MT2A*, and *MT2B* subfamily genes in *B. napus* (Figure 6). *BnaMT1* was expressed at the highest level in siliques of 30D, except for roots and leaves, whereas *B. napus* *MT2A* genes (with four members; *BnaMT2A*, *BnaMT2C*, *BnaMT2H* and *BnaMT2I*) and *MT2B* genes (with six members; *BnaMT2B*, *BnaMT2D*, *BnaMT2E*, *BnaMT2F*, *BnaMT2G* and *BnaMT2J*; Figure 2) showed variable expression patterns. For example, *BnaMT2A* and *BnaMT2H* were preferentially expressed in buds, and *BnaMT2G* was expressed at low levels in all organs (Figure 6). These differences may be attributed to concentrations and species differences in future works. However, *B. napus* *MT3* and *MT4* subfamily genes shared similar expression patterns with *AtMT3* and *AtMT4* [14]. *B. napus* *MT3* genes were mainly expressed in stems, roots, and leaves, and *B. napus* *MT4s* were primarily expressed in developing seeds (Figure 6). The expression patterns of these *BnaMTs* revealed by RT-qPCR corresponded well with the patterns obtained by transcriptome analysis under normal conditions, although there were differences among *B. napus* varieties (Figure 7). Although no comprehensive heavy metal tolerance mechanisms have been uncovered in *Brassica*, distinctive expression patterns were identified among the *B. napus* MT family members in this work, laying the foundation for investigating the biochemical and physiological functions of MTs in plants.

Heavy metal (Cu, Cd, and As) pollution in agricultural soils has become a critical problem affecting crop production and quality. The absorption of these heavy metals by plants plays an important role in the entry of these metals into the food chain [42]. Further, MTs have been shown to play an important role in metal homeostasis and tolerance in plants [4,6,9,10,21,26,33,34]. Strikingly, *Brassica* plants exhibit efficient heavy metal uptake and translocation, as well as a high tolerance to heavy metals [31,42], and several MT genes in *Brassica* have been reported, especially in Indian
mustard (*Brassica juncea* L.) [23,27,33,53]. Recently, three *B. rapa* metallothionein genes (*BrMT1–3*) displayed differential expression levels under various exogenous stress factors [54], and MT-like, protein-encoding gene transcription was obviously induced in roots and leaves of *B. napus* under As treatment [55]. Here, we investigated the expression profiles of *BnaMT* family genes in *B. napus* under normal conditions and As$^{3+}$ stress. The *BnaMTs* were obviously induced by the As$^{3+}$ treatments, which is in accordance with findings that MTs are involved in the chelation and sequestration of heavy metals [3,4,55]. Like other plant MT genes [4,41,54,55], however, these genes also had different expression profiles in different *B. napus* varieties and in different tissues, with high expression levels in cotyledons and low expression levels in hypocotyls, such as *BnaMT1*, *BnaMT2A*, *BnaMT2B*, *BnaMT2C*, *BnaMT2F*, *BnaMT2J*, *BnaMT3B*, and *BnaMT3C* (Figure 7). These results suggest that hypocotyls might merely be involved in the transport of heavy metal ions, but that these ions accumulate in roots and cotyledons. In addition, *BnaMT3C* was obviously increased in the roots and hypocotyls of B33 and B34, but the higher expression levels of B93 and B113 in cotyledons (Figure 7), which comply with the finding that B33 and B34 exhibited better growth than B93 and B113 under As$^{3+}$ treatment (Figure S2), indicate that they play crucial roles in the response to As$^{3+}$ stress in *B. napus*. Our results provide important information for further functional studies of MT family genes in *B. napus*.

4. Materials and Methods

4.1. Identification of MT Family Genes in Brassica

The amino acid sequences of MTs from the Arabidopsis Information Resource (TAIR10) database (ftp://ftp.arabidopsis.org) were used as queries for the BLASTp analysis against the whole genome sequences in the *Brassica* database [56]. The candidate sequences with $E$-values $\leq 1 \times 10^{-20}$ were identified and confirmed using the Hidden Markov model (HMM) searches program (HMMER v3.0, http://hmmer.janelia.org/), and the BLAST analysis of the MTs was performed against a *Brassica* protein database constructed using Geneious v4.8.5 software (http://www.geneious.com/, Biomatters, Auckland, New Zealand). The coding sequences (CDS) of the MTs were identified by BLASTn searches against the *Brassica* genome database. The candidate proteins were named using the species abbreviation of the source organism (italicized), the gene family name, and the positions in the subtribe, e.g., *AtMT1B* and *BnaMT1A*. Physicochemical properties, including the molecular weight (kDa), isoelectric point (pI), and the grand average of hydropathy (GRAVY) value of each deduced protein were determined using the online ExPASy-ProtParam tool (http://web.expasy.org/protparam/).

4.2. Multiple Sequence Alignment and Phylogenetic Analysis of MTs in Brassica

The deduced amino acid sequences of MT proteins from *A. thaliana* and various *Brassica* species, including *B. rapa*, *B. oleracea*, *B. napus*, *B. juncea*, and *B. nigra*, were subjected to multiple protein sequence alignment using the ClustalW software with default settings [57]. To illustrate the evolutionary relationships of MTs in *Brassica*, a neighbor-joining (NJ) phylogenetic tree was generated with the MEGA v6.0 program (Tokyo Metropolitan University, Tokyo, Japan) using the JTT+I+G substitution model and a bootstrap test with 1000 replicates [58]. The phylogenetic trees were visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

4.3. Conserved Motif Recognition and Gene Structure Analysis

The CDS of the MTs from the *Brassica* species were retrieved based on their protein sequences, and the corresponding genomic sequences were extracted from the *Brassica* genome sequences. The exon–intron structures of the MTs were analyzed online using the Gene Structure Display Server (GSDS v2.0, http://gsds.cbi.pku.edu.cn/index.php). Conserved motifs were identified using Multiple Expectation Maximization for Motif Elucidation (MEME v4.12.0, http://meme-suite.org/tools/meme) with the following parameters: number of repetitions, any; maximum number of motifs, 15; and...
optimum width of each motif, between 6 and 300 residues [59]. Each motif with an $E$-value < $1 \times 10^{-10}$ was retained for motif detection.

4.4. Chromosomal Locations of MT Family Genes in B. napus

The MT family genes were mapped to the rapeseed chromosomes according to their physical distances in the GFF genome files, which were downloaded from the B. napus genome database (http://www.genoscope.cns.fr/brassicanapus/) [43]. A map of the chromosomal locations of the MTs was constructed using MapChart v2.0 (https://www.wur.nl/en/show/Mapchart.htm) [60].

4.5. Plant Materials and Metal Stress Treatments

B. napus seeds were collected from the Rapeseed Engineering Research Center of Southwest University in Chongqing, China (CERCR). Fifty healthy seeds were selected and soaked in a dish (the diameter was 90 mm) containing deionized water for 24 h. Then morphologically uniform seedlings were selected and plugged into a hydroponic system with a float tray (60 cm $\times$ 40 cm $\times$ 10 cm) for 7 days. Here, the seedlings were exposed to distilled water and 35 $\mu$M As$^{3+}$ solutions, respectively. Meanwhile, they were cultivated under long-day conditions (16 h light/8 h dark, 5000 Lux) at 25 °C. After 7 days, the whole roots, hypocotyls, and cotyledons were sampled to analyze the MT gene expression patterns; the tissues were snap frozen in liquid nitrogen and stored at $-80 \degree$C prior to total RNA extraction. All experiments were repeated three times.

4.6. Total RNA Extraction and RT-qPCR Analysis

As the B. napus cultivars B33 and B34 grow better than B93 and B113 under heavy metal treatment (Figure S2), they were therefore used for expression analysis. Total RNA was isolated from the samples using a DNAway RNA Mini-Prep Kit (Sangon Biotech, Shanghai, China). For the tissue-specific expression analysis, RNA was extracted from the roots, hypocotyls, and cotyledons and pretreated with gDNA Eraser (Takara, Dalian, China). Subsequently, 1 $\mu$g of the total RNA was used to synthesize first-strand cDNA with an RNA PCR Kit (AMV) Ver. 3.0 (Takara, Dalian, China). The cDNA was subjected to RT-qPCR analysis using SYBR Premix Ex Taq II (Takara, Dalian, China) on a Bio-Rad CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [61]. BnACTIN7 (EV116054) was employed as a reference gene to normalize MT gene expression levels via the $2^{-\Delta\Delta Ct}$ method [62]. All experiments were performed with three technical replicates, and the values represent the average ± standard error (SE). The specific primer sequences used in this study were obtained from the qPCR Primer Database [63] and are listed in Supplementary Table S4.

4.7. Statistical Analysis

All experiments were repeated three times (three biological replicates). All data were statistically analyzed using the Student’s $t$-test with the statistical analysis software package SPSS v15.0 (IBM Corp, Armonk, NJ, USA).

5. Conclusions

In this study, we identified 60 MTs from A. thaliana and five Brassica species. The phylogenetic analysis showed that all MT family genes are closely associated with the AtMTs. Genome-mapping analysis revealed high levels of synteny throughout the genome due to whole genome duplication and segmental duplication events. In addition, all 16 BnaMTs were induced by heavy metal stress, especially in cotyledons versus roots and hypocotyls. Finally, BnaMT3C might improve the response to As$^{3+}$ stress in B. napus. Our results provide a basis for the further functional analysis of the molecular functions of MT family genes in B. napus.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/8/2181/s1.
Author Contributions: C.Q. (Cunmin Qu) and J.L. conceived and designed the experiments; Y.P. and M.Z. conducted the experiments; S.W., G.M. and X.H. collected and analyzed the data; Y.L., C.Q. (Cailin Qiao), R.W. and X.X. carried out the experiments and performed the software analyses; K.L. and C.Q. (Cunmin Qu) wrote the manuscript; and C.Q. (Cunmin Qu) and J.L. reviewed the manuscript.

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