

Article

Genome-Wide Identification and Evolution-Profiling Analysis of the GDSL Gene Family in Apple (*Malus × domestica*)

Jiawei Yuan ^{1,†}, Zhejun Liang ^{1,†}, Lu Wang ¹, Fangjuan Chang ¹, Mengni Chen ¹, Peng Dong ¹, Jian Zhang ¹, Yaoyao Li ¹ , Hui Wang ¹, Zhanbei Zhang ¹, Xiaoling Zhang ¹, Yongshan Li ¹ , Tong Miao ^{2,*} and Jiancheng Zhang ^{1,*}

¹ Cotton Research Institute, Shanxi Agricultural University, Yuncheng 044000, China; yjwsxnky@126.com or yuanjiawei@sxau.edu.cn (J.Y.); liangzhejun@sxau.edu.cn (Z.L.); wangl@sxau.edu.cn (L.W.); fangjuanch@sxau.edu.cn (F.C.); mengni225@sxau.edu.cn (M.C.); dongpeng@sxau.edu.cn (P.D.); zhangjian@sxau.edu.cn (J.Z.); liyaoyao@sxau.edu.cn (Y.L.); hwang328@sxau.edu.cn (H.W.); zhangzhanbei@sxau.edu.cn (Z.Z.); zhangxiaoling2@sxau.edu.cn (X.Z.); sxysli@sxau.edu.cn (Y.L.)

² Sci-Tech Information and Strategic Research Center of Shanxi Province, Taiyuan 030001, China

* Correspondence: sxkjmt@163.com (T.M.); zhangjc@sxau.edu.cn (J.Z.); Tel.: +86-180-1015-2597 (J.Z.)

† These authors contributed equally to this work.

Abstract: Background/Objectives: GDSL lipase is crucial for plant growth and the response to environmental stress. While GDSL gene families have been identified in some plants, a comprehensive genome-wide identification and analysis in *M. domestica* is lacking. Methods: In this study, we identified 131 MdGDSLs belonging to four subfamilies, distributed across 17 chromosomes in the *M. domestica* genome. Results: The molecular weights (MWs) of these MdGDSLs range from 14.44 to 83.26 kDa, with isoelectric points (PI) spanning from 4.32 to 10.09. Members within the same subgroup exhibit similar gene structures and conserved motifs. Furthermore, whole genome duplication (WGD) significantly contributes to the evolution of the MdGDSL gene family. The promoter regions of MdGDSL genes contain numerous cis-elements associated with abiotic stress and hormone responses. Additionally, RNA-seq data indicate that MdGDSL genes respond to high concentrations of CaCl₂ and cold stress treatments. Conclusions: This study provides a theoretical foundation for further investigation into the mechanisms underlying growth, development, and lipid metabolism in response to stress in *M. domestica*.

Keywords: *M. domestica*; GDSL lipase gene; systematic evolution; expression analysis



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1. Introduction

GDSL lipase is a distinctive hydrolase characterized by the presence of the GDSL motif. This enzyme is capable of hydrolyzing a variety of substrates, including phospholipids, sulfur esters, aromatic esters, and amino acids [1]. The first report on GDSL lipase was published by Upton and Buckley in 1995 [2]. Research on GDSL lipase has primarily concentrated on animals and microorganisms, with relatively few studies conducted on its role in plants [3]. Current studies have demonstrated that GDSL lipase plays a significant biological role in various physiological activities, including plant growth and development [4,5], stress responses [6–8], and oil lipid metabolism [9]. Consequently, plant GDSL lipase has garnered considerable attention from researchers in recent years when compared to other molecules cited to have a biological role in plant growth, stress responses, and oil lipid metabolism.

The primary structure of GDSL lipase comprises five conserved domains and catalytic amino acid residues (Ser, Gly, Asn, and His) located within domains I, II, III, and V. The GDSL motif, present in domain I, features Ser as a nucleophile that facilitates proton transfer to oxygen anion holes. Gly and Asn contribute additional protons for the oxygen anion hole, while His residues enhance the nucleophilicity of Ser by deprotonating its hydroxyl group [1,10]. In 2001, the crystal structure of TEP-1, a GDSL lipase from *E. coli*, was reported as a globular protein characterized by multiple α -helices and β -folds [11]. Although the crystal structure of plant-derived GDSL lipase has not yet been reported, studies utilizing homology modeling and other methods have been conducted. For instance, Chepyshko et al. employed the PHYRE methods to analyze the protein structure of rice GDSL lipases, predicting six alpha helices and six beta folds [12]. The protein structure of the walnut GDSL lipase family exhibits varying numbers of α -helices and β -folds [13].

Current studies have demonstrated that GDSL lipases play key roles in various physiological activities, including plant growth and development [4,5], stress responses [6–8], and lipid metabolism [9]. For instance, the OsGER1 is induced by jasmonic acid and light, exerting a negative regulatory effect on germ elongation in rice [14]. Notably, the overexpression of GhGLIP in *Arabidopsis thaliana* has been shown to influence seed length and fresh weight [15]. Furthermore, GDSL1 is essential for the extracellular deposition of cuticle polyester in tomatoes; its loss of function leads to a thinner fruit epidermis [16].

Regarding abiotic stress, overexpression of the AtLTL1 could enhance *Arabidopsis* resistance to salt stress, with the gene being induced by salicylic acid [17]. Moreover, the expression of the GDSL genes were induced by various stress factors, including drought, high salinity, injury, and salicylic acid [18]. Recent studies have shown that GDSL lipase is involved in the accumulation of the waxy layer in the epidermis of sorghum, thereby reducing water loss and improving the plant's drought resistance [19]. Zhao et al. conducted proteomic analysis of *Avena sativa* seedlings under alkali stress and found that a large number of resistant proteins such as GDSL lipase accumulated in the roots of oat that were resistant to alkali stress [20]. Chiconato et al. conducted proteome analysis of sugarcane seedlings under salt treatment and found that the expression of GDSL lipase in salt-treated tolerant varieties was three times that of the control group [8]. It is concluded that GDSL lipase plays an important role in plant abiotic stress response. Recently, the rapid advancement of high-throughput sequencing technology has led to the sequencing of an increasing number of plant genomes, thereby providing a foundation for a comprehensive understanding of the biological functions of the GDSL lipase gene family. For instance, 105, 114, 121, 194, 159, and 103 GDSL members have been identified in *A. thaliana*, *O. sativa*, *B. rapa*, *G. max*, *D. villosum*, and *Z. mays*, respectively [12,21–25]. These data indicate that GDSL lipases are abundant in plants and may serve various biological functions; however, the regulatory mechanisms of the GDSL gene family in *M. domestica* are still poorly reported.

To gain insight into the role of the GDSL gene family in *M. domestica*, a comparative analysis of the GDSL gene family was performed, including the molecular characteristics, conserved motifs, phylogenetic classifications, chromosome distribution and synteny analysis. This provides a theoretical basis for the further study of its mechanism in *M. domestica* growth, development and stress response.

2. Materials and Methods

2.1. Identification of GDSL Gene Family in *M. domestica*

The genome data and General Feature Format (GFF) of the *M. domestica* were obtained from the Ensembl Plants database (<https://plants.ensembl.org/index.html> (accessed on 7 October 2024)) and Phytozome database (<https://phytozome-next.jgi.doe.gov/> (accessed on 10 October 2024)) [26]. The AtGDSL and OsGDSL protein sequences were downloaded

from the TAIR database (<https://www.arabidopsis.org/> (accessed on 12 October 2024)) and the Rice Genome Annotation Project database (<https://rice.uga.edu/> (accessed on 16 October 2024)), respectively. The hidden Markov Model (HMM) files for PF00657 were downloaded from the InterPro database (<https://www.ebi.ac.uk/interpro/> (accessed on 18 October 2024)) [27]. Then, we utilized HMMER 3.0 software (E-value $\leq 1 \times 10^{-5}$, similarity $> 50\%$) to identify MdGDSL proteins [28]. Additionally, we employed the BLASTP method [29] to search for protein sequences in *M. domestica* using AtGDSL and OsGDSL as queries (E-value $\leq 1 \times 10^{-5}$, similarity $> 50\%$) [29]. All candidate MdGDSL protein sequences were subsequently analyzed to confirm the presence of the GDSL domain as described previously [21,23]. The longest transcript was filtered using the R package seqfinder (<https://github.com/yueliu1115/seqfinder> (accessed on 20 October 2024)).

2.2. Phylogenetic Analysis of GDSL Genes in *M. domestica*

The Muscle software (v3.8.1551) [30] was utilized to conduct multiple sequence alignment of the MdGDSL, AtGDSL and VvGDSL proteins. Subsequently, a maximum likelihood (ML) phylogenetic tree was constructed using the IQ-TREE software (2.0.3) with a bootstrap setting of 1000 [31]. The visualization of the phylogenetic tree was performed using the R package ggtree (3.10.0) [32].

2.3. Duplication Event Analysis of MdGDSL Family Members

The chromosomal distribution of MdGDSL genes was derived from the genome annotation data of *M. domestica* (GFF). The MCScanX software (1.0.0) was employed for gene duplication and collinearity analysis [33]. To ascertain the types of gene duplication, we utilized the program duplicate_gene_classifier. The identified gene duplication types include singleton, dispersed repeat (DSD), proximal repeat (PD), tandem repeat (TD), and genome-wide repeat (WGD) [33]. Subsequently, JCVI software (1.4.16) was used for interspecific collinearity analysis and visualization [34]. KaKs_Calculator software (2.0) was applied to calculate the synonymous substitution rate (Ks), non-synonymous substitution rate (Ka), and evolutionary ratio (Ka/Ks) of duplicate TPS gene pairs [24].

2.4. Analysis of Cis-Elements in the Promoter of MdGDSL Genes

The 2000 bp promoter sequences of MdGDSL family genes were extracted from the genomic files (GFF). The website of PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/> (accessed on 25 October 2024)) was utilized to conduct a statistical analysis of cis-element prediction for each gene promoter [35].

2.5. MdGDSL Protein Interaction Network and Its Functional Annotation

To explore the regulatory network of MdGDSL protein, we used AraNet2 database to predict the interaction network of MdGDSL protein. In addition, we annotated the GO and KEGG results of *M. domestica* GDSL using eggNOG-mapper database, and used R software (4.3.0) for statistics and visualization.

2.6. Expression Pattern Analysis of MdGDSL Genes Using RNA-Seq

To investigate the expression pattern of the MdGDSL gene under abiotic stress. RNA-seq in calcium chloride stress and low-temperature treatment was performed on the Illumina NovaSeq 6000 (Illumina, San Diego, California, USA; Illumina (China) Scientific Co., Ltd. Shanghai, China), from which 150 bp paired-end reads were generated. The above work was carried out in Biomarker Technologies (Peking, China). The transcriptome data were aligned and quantified using the genome alignment software HISAT2 (2.2.0) and featureCounts (1.6.4) for subread regions. The R package Pheatmap (1.0.12) was employed

to visualize the resulting data. All fastq files have been uploaded to the SRA database under the accession number PRJNA1204483.

2.7. Plant Materials and Treatments

Ca^{2+} plays a crucial role in plant physiology, functioning as a second messenger in cellular signal transduction, stress responses, and structural stability. It regulates plant responses to environmental stresses such as low temperature, salt stress, drought, and mechanical damage while also improving plant resilience, yield, and fruit quality. This study aims to elucidate whether members of the GDSL gene family are differentially affected by Ca^{2+} application under varying treatment conditions. The experiment was conducted at the fruit tree planting demonstration base in Yancun ($110^{\circ}42'28''$ E, $35^{\circ}26'2''$ N), located in Nanzhang Township, Wanrong County, Yuncheng City, China. The site has an average annual temperature of 12.9°C and an average annual precipitation of 486.0 mm. The test material consisted of nine-year-old Fuji apple trees, and healthy trees with similar growth characteristics were selected from the experimental area, which had a row spacing of $4\text{ m} \times 1.5\text{ m}$. A completely randomized experimental design was employed to investigate the effects of Ca^{2+} treatment under different conditions. Two experimental setups were conducted: one focused on the response of floral tissues to low-temperature stress combined with Ca^{2+} application, and the other examined the effect of foliar-applied calcium on apple leaves.

For the floral tissue experiment, four treatments were applied: (T1) flowers without low-temperature stress (control), (T2) flowers subjected to low-temperature stress, (T3) flowers subjected to low-temperature stress after water spraying, and (T4) flowers subjected to low-temperature stress after spraying with 20 mmol/L CaCl_2 . Each treatment had three replicates, with three apple trees per replicate. Field spraying was performed on 26 March 2024, ensuring that water droplets formed on the flower organs without dripping. Sampling was conducted on 29 March 2024, with ten similarly shaped flower shoots collected per replicate for each treatment. To simulate field conditions, a low-temperature climate chamber at the Shanxi Fruit Tree Seedling Breeding and Plant Protection Technology Innovation Center was used. The temperature was gradually reduced from 0°C by 1°C every 30 min, reaching a minimum of -4°C , with sub-zero conditions maintained for 5 h. For the foliar calcium application experiment, five treatments were established: 0.5% CaCl_2 (Ca1), 1.0% CaCl_2 (Ca2), 4.0% CaCl_2 (Ca3), 6.0% CaCl_2 (Ca4), and a non-fertilized control (CaCK). Each treatment had three replicates, with three apple trees per replicate. Calcium fertilizer was sprayed on 6 September 2023, ensuring uniform coverage on both leaves and fruits without dripping. Leaf samples were collected on 22 September 2023 by selecting three healthy leaves from different orientations and the same height per tree. Samples were immediately frozen in liquid nitrogen for further analysis.

2.8. Total RNA Extraction and RT-qPCR Analysis

The design of specific primers for *MdGDSL* genes in this study was accomplished using the primer 5.0 software (Supplemental Data S4). To extract total RNA, the FastPure Plant Total RNA Isolation Kit (Vazyme, RC401; Vazyme, Nanjing, China) was utilized. The synthesis of cDNA was carried out with the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, R211-01; Vazyme, Nanjing, China). The reaction mixture for each RT-qPCR consisted of 5 μL of $2 \times \text{ChamQ SYBR qPCR Green Master Mix}$, 3 μL of the cDNA sample, 1 μL of forward primer (at a final concentration of 10 μM), and 1 μL of reverse primer (also at a final concentration of 10 μM), reaching a total volume of 10 μL . The amplification protocol for RT-qPCR included an initial step at 95°C for 5 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

3. Results

3.1. Genome-Wide Identification of the MdGDSL Genes

A BLAST (2.9.0+) search was conducted using the *A. thaliana* GDSL and *O. sativa* GDSL protein sequences to identify the GDSL protein sequences and conserved domains in *M. domestica*, ultimately identifying 131 MdGDSL members (Supplemental Data S1). Following the identification of the *M. domestica* GDSL family members, analyses were performed on several parameters, including amino acid count, molecular weight, isoelectric point, and hydrophilicity (Supplemental Data S1). The number of amino acids in *M. domestica* GDSL proteins ranges from 131 to 767, with an average length of 353 amino acids (Supplemental Data S1). The molecular weight varies from 14.44 to 83.26 kDa, with an average molecular weight (MW) of 38.85 kDa. The isoelectric point (PI) spans from 4.32 to 10.09, with an average of 7.51. Additionally, the mean coefficient of overall hydrophilicity ranges from -0.57 to 0.34 .

3.2. Phylogenetic Analysis of the MdGDSL

To investigate the phylogenetic relationships among MdGDSL proteins, we constructed phylogenetic trees utilizing 132 MdGDSL, 111 AtGDSL and 105 VvGDSL

Protein sequences. The results revealed that these GDSL proteins can be categorized into four distinct subgroups: subgroup I contains 87 members, subgroup II consists of 43 members, subgroup III includes 79 members, and subgroup IV comprises 166 members, making it the largest branch (Figure 1). Subgroup I follows with 86 GDSL members, while subgroup II is the smallest, with only 43 GDSL members. Additionally, subgroup I encompasses 42 AtGDSL, 31 MdGDSL, and 14 VvGDSL; subgroup II comprises 17 AtGDSL, 16 MdGDSL, and 10 VvGDSL; subgroup III contains 24 AtGDSL, 31 MdGDSL, and 24 VvGDSL; and subgroup IV includes 28 AtGDSL, 53 MdGDSL, and 81 VvGDSL (Figure 1).

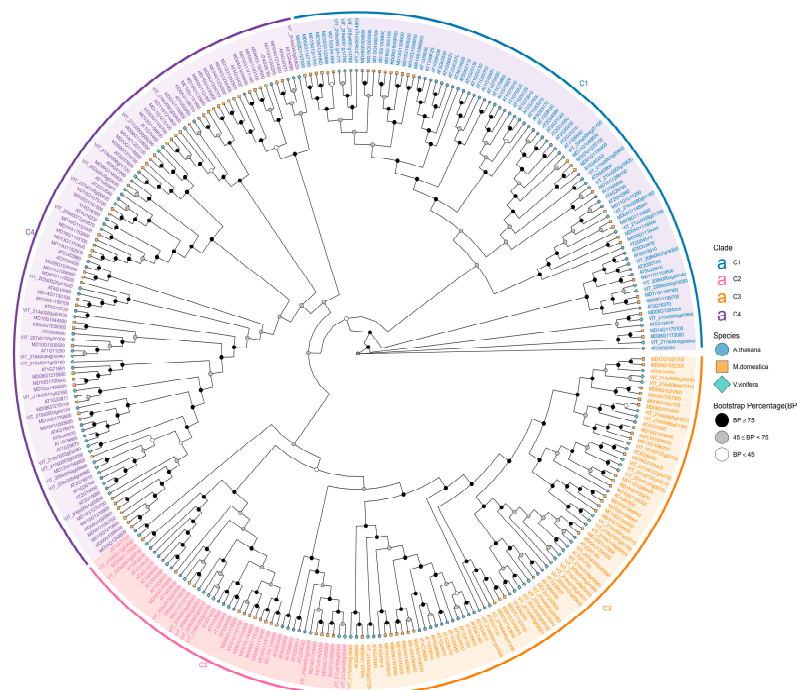


Figure 1. The evolutionary examination of the 347 GDSL protein sequences identified from *M. domestica*, *V. vinifera* and *A. thaliana* were conducted. These 347 GDSL proteins were categorized into four distinct subgroups, labeled C1, C2, C3, and C4. The evolutionary phylogeny was analyzed utilizing IQ-TREE software (2.0.3), employing the maximum likelihood (ML) method with bootstrap values derived from 1000 replicates. The phylogenetic tree was visualized through the use of the R package ggtree (3.10.0).

3.3. Conserved Motifs, Conserved Domain and Gene Structure of the MdGDSL

The diversity of gene structures can provide insights into the evolutionary processes of gene families. Consequently, we analyzed the conserved domains, protein conserved motifs, and gene structures of the MdGDSL members. The analysis of conserved domains revealed that most MdGDSL members possess a single “Lipase_GDSL” domain. However, some MdGDSL proteins, such as Md13G1152200 and Md05G1128700, contain two “Lipase_GDSL” domains. Notably, Md15G100900 was found to have an “RVT_2” domain in addition to a “Lipase_GDSL” domain (Figure 2), suggesting that this gene may have a specialized function.

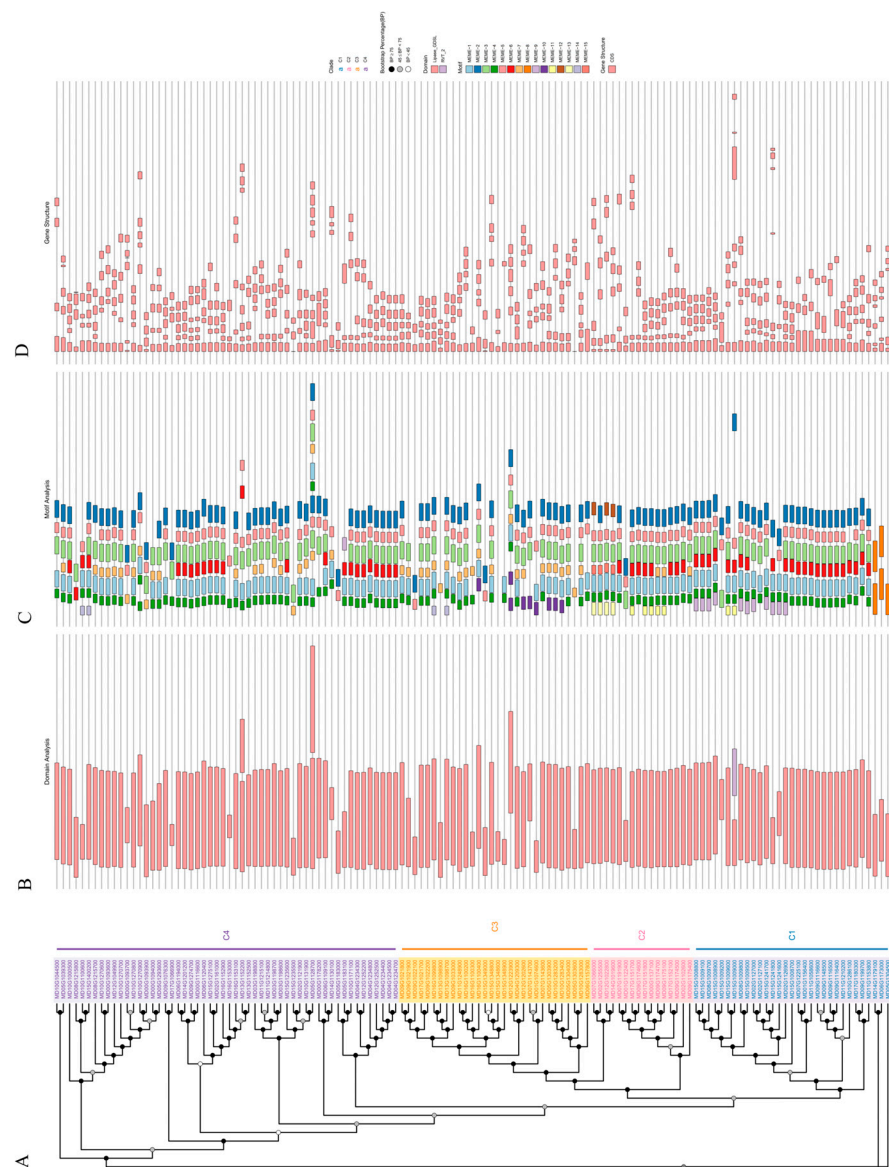


Figure 2. Analysis of phylogenetic relationships, conserved domains, motifs, and gene structures related to GDSL in *M. domestica*. (A) Phylogenetic tree depicting GDSL proteins from *M. domestica*. The evolutionary analysis was performed using IQ-TREE software (2.0.3) (method: maximum likelihood (ML); bootstrap values: 1000 iterations). (B) The conserved domains present in GDSL protein sequences found in *M. domestica*. (C) Distribution of conserved motifs across GDSL proteins in *M. domestica*, with 15 putative motifs represented in various colored boxes. (D) Gene structure associated with GDSL genes in *M. domestica*.

Conserved motifs are essential for protein function. We employed the MEME tool to identify 132 motifs within MdGDSL proteins, subsequently isolating 15 consensus motifs, which we designated as MEME1-15. Among these, conserved motifs 1, 2, 3, 4, and 5 of the GDSL domain are present in nearly all MdGDSL proteins (Figure 2). Additionally, we noted that MdGDSL members within the same subpopulation exhibit similar conserved motifs. Notably, certain MdGDSL members possess only a single conserved motif; for example, Md14G1179100, Md06G1173000, and Md05G1304500 each contain only Motif 15, while Md05G1303600 contains solely Motif 7. In contrast, Motif 6 is exclusively found in subgroups I, II, and IV, whereas Motif 7 is unique to subgroup III, suggesting functional differentiation among these subgroups during evolution.

Gene structure analysis revealed that the majority of MdGDSL members possess four to five exons, whereas Md13G1152200 contains seven exons and Md05G1128700 has nine exons. Additionally, Md00G1093700, Md10G1094000, Md14G1179100, and Md06G1173000 each have six exons (Figure 2). Among members with the same number of exons, there is considerable variation in exon length and distribution. Furthermore, genes that are more closely related tend to exhibit greater similarity in their genetic structure and length.

3.4. Gene Duplication of MdGDSL Genes

To determine the distribution of the MdGDSL gene across chromosomes, we analyzed the location of the MdGDSL genes using a GFF file. Our results indicated that 124 MdGDSL genes were distributed across chromosomes 1 to 17. However, seven genes (MD00G1116600, MD00G1178200, MD00G1093700, MD00G1093900, MD00G1137100, MD00G1094000, and MD00G1093600) could not be assigned to any chromosome (Figure 3). While all 17 *M. domestica* chromosomes contain some MdGDSL genes, their distribution is uneven. The highest concentration of MdGDSL genes is found on chromosome 15, followed by chromosome 9. In contrast, chromosomes 1 and 3 each harbor only three MdGDSL genes (Figure 3).

To elucidate the mechanism of MdGDSL expansion during evolution, we conducted a collinearity alignment of the *M. domestica* genome and examined the collinearity of GDSL genes in the genomes of *A. thaliana* and *V. vinifera*. As illustrated in Figure 3, we identified 35, 16 and 25 GDSL TD gene pairs in *M. domestica*, *A. thaliana*, and *V. vinifera*, respectively, as well as 42, 12, and 5 WGD GDSL gene pairs. Notably, *M. domestica* contain the highest number of TD and WGD GDSL genes compared to *A. thaliana* and *V. vinifera* (Figure 3). Interestingly, we observed that the TD genes in *M. domestica* predominantly belong to subgroup I, whereas those in *A. thaliana* are primarily found in subgroup III. In contrast, the WGD genes in *M. domestica* are mainly classified in subgroup IV, while the genome-wide repeats in *A. thaliana* are predominantly located in subgroup II. Collectively, these findings suggest that WGD is the principal driver behind the substantial expansion of GDSL genes in the *M. domestica* genomes, while in *A. thaliana* and *V. vinifera*, the expansion of the GDSL gene family is chiefly influenced by TD.

To evaluate the pressure of natural selection on the GDSL gene family in these species, we computed the Ka/Ks ratio, which represents the ratio of non-synonymous substitutions per non-synonymous site (Ka) to synonymous substitutions per synonymous site (Ks). The outcomes indicated that the Ka/Ks ratio for all GDSL duplication genes in *M. domestica*, *A. thaliana*, and *V. vinifera* was less than 1, except MD15G1348800/MD15G1348900 and VIT_218s0001g02760.1/VIT_218s0001g02770.1 (Supplemental Data S2), indicating that most of the GDSL genes in these species may have undergone purification selection during evolution.

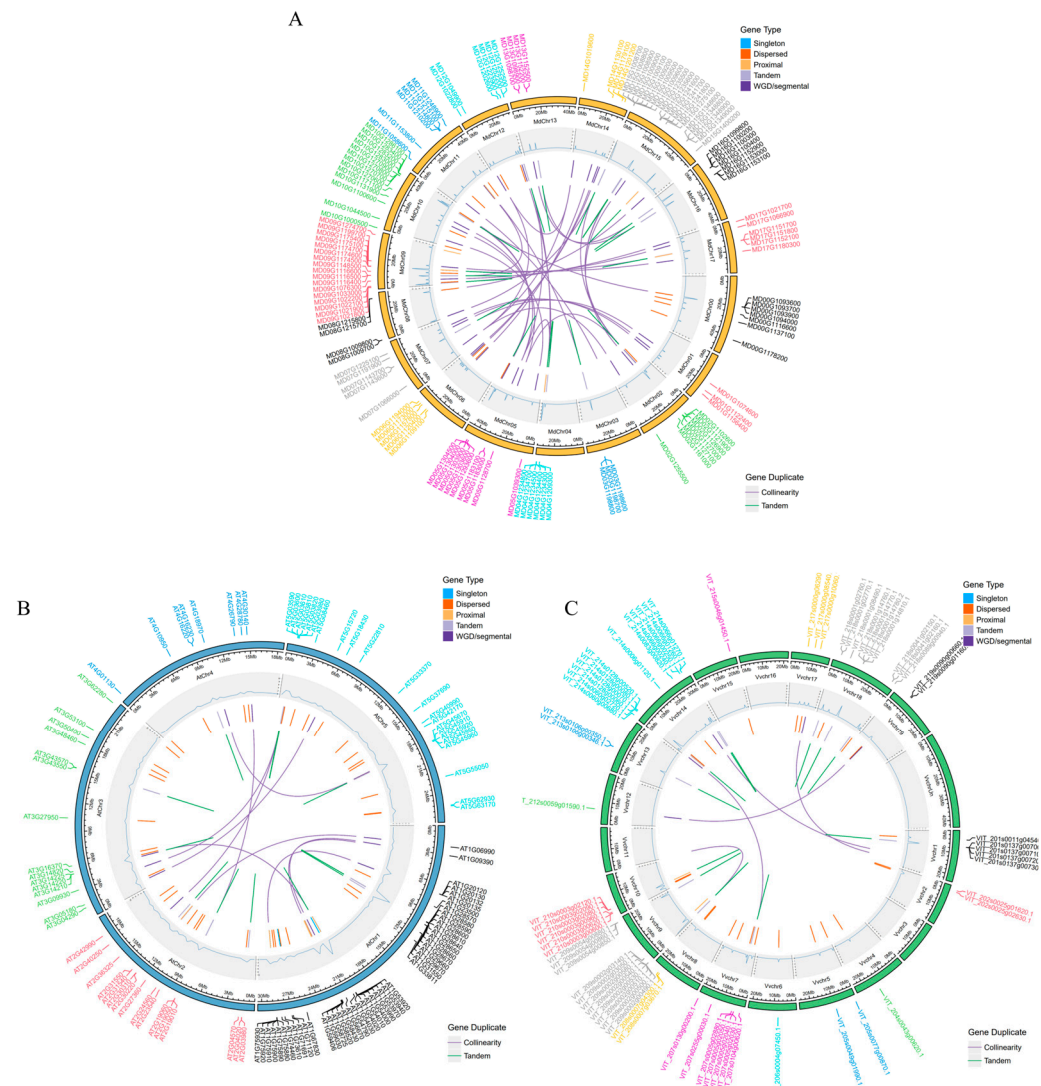


Figure 3. Analysis of gene duplication events for GDSL genes in *M. domestica* (A), *A. thaliana* (B) and *V. vinifera* (C). The TD and WGD gene names are highlighted in green and purple, respectively.

3.5. Collinearity Analysis

To elucidate the evolutionary mechanisms of the *M. domestica* GDSL gene family, we examined the collinearity relationships between *M. domestica* and both Arabidopsis and grape. Our results indicated the presence of 42 collinear gene pairs between *M. domestica* and *A. thaliana*, and 27 collinear gene pairs between *M. domestica* and *V. vinifera* (Figure 4). These genes may have been inherited from a common ancestor, suggesting a closer evolutionary relationship between *M. domestica* and *V. vinifera* than between *M. domestica* and *A. thaliana*. Additionally, collinear events predominantly occurred on chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, and 17 (Figure 4). These genomic regions may be hot spots for the evolution of GDSL gene families in *M. domestica*. Furthermore, eight MDGDSL genes (MD08G1009800, MD09G1116400, MD09G1199700, MD10G1270900, MD10G1286100, MD11G1210200, MD12G1202600, MD14G1179100, and MD15G1009600) exhibited collinearity genes from both *A. thaliana* and *V. vinifera*. Notably, some MdGDSL genes display collinearity with at least two genes; for instance, MD11G1210200 is collinear with VIT_212s0059g01590.1 and VIT_219s0090g01160.16, while MD08G1009800 is collinear with AT1G20132.1 and AT1G75900.1. These findings suggest that these genes may have been functionally conserved during evolution.

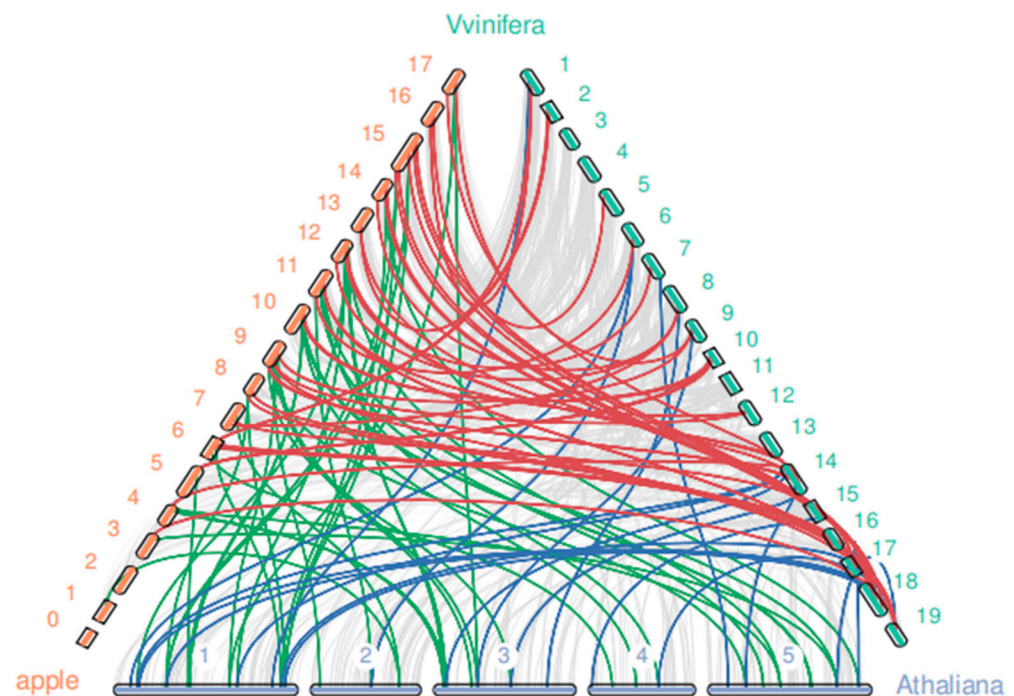


Figure 4. Analysis of synteny among *M. domestica*, *V. vinifera* and *A. thaliana* was conducted. The collinear blocks associated with *M. domestica*, *V. vinifera* and *A. thaliana* are represented by red, green and blue lines, respectively.

3.6. Analysis of Promoter Elements

Transcription levels of genes are regulated by the binding of cis-acting elements to transcription factors. To explore the functions of GDSL family members during *M. domestica* growth and development, as well as in response to hormonal, biological, and abiotic stresses, we utilized Plant-CARE, an online tool, to predict cis-acting elements within the 2.0 kb promoter sequence of the MdGDSL genes. The predictive analysis revealed the presence of 21 cis-acting elements (Figure 5), including 5 stress response elements (ARE, as-1, MBS, LTR, and WUN-motif), 6 hormone response elements (TGA-element, ABRE, ABRE3a, ABRE4, CGTCA-motif, and TGACG-motif), 5 elements related to plant growth and development (CAT-box, TATA-box, ERE, AAGAA-motif, and O2-site), and 5 light-responsive elements (CAAT-box, Box-4, G-box, GT1-motif, and G-Box). Notably, the promoter regions of the MD04G1234800, MD12G1252500, and MD01G1074600 genes contained 12, 10, and 10 ABRE cis-regulatory elements, respectively, suggesting that these genes may play significant roles in ABA signaling pathways. MD00G11093900 and MD12G1126900 genes contained eight and six LTR cis-regulatory elements (Figure 5), respectively, suggesting that these genes may play significant roles in low-temperature signaling pathways. Additionally, the promoter region of the MD04G1234300 gene contains five WUN-motif cis-regulatory elements. These findings indicate that the MdGDSL genes have functionally differentiated during evolution. In conclusion, cis-acting elements associated with abiotic stress, hormones, light, and growth and development are widely present in the promoter of the MdGDSL genes. The results indicated that GDSL may play a role in *M. domestica* growth, abiotic stress, hormones and light.

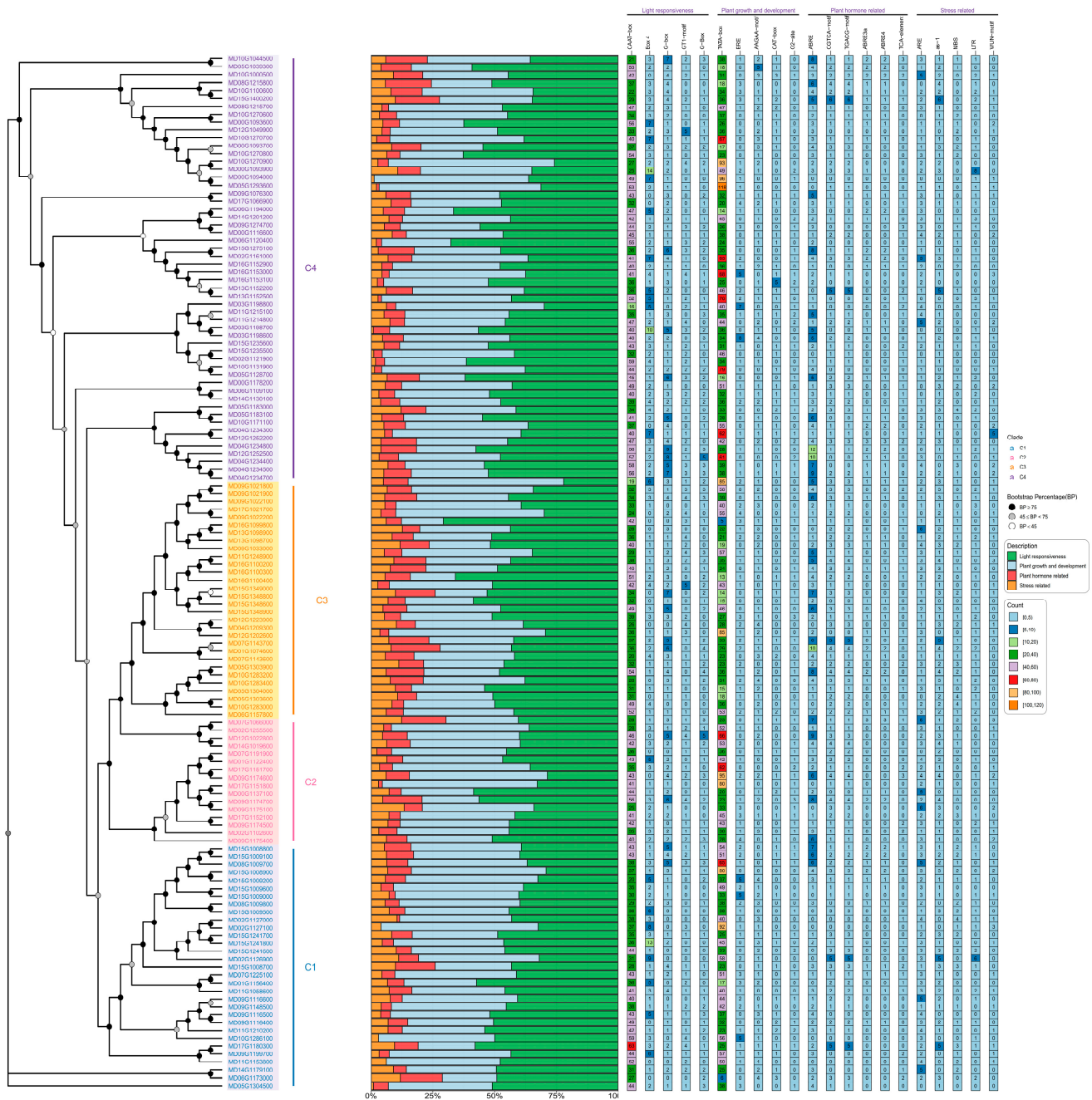


Figure 5. The prevalence of cis-acting elements within the GDSL gene family in *M. domestica* was examined. Cis-acting elements were identified in the 2000 bp promoter regions of MdGDSL genes. The promoter sequences, each measuring 2000 bp, for the MdGDSL family were retrieved from genomic files (GFF). A statistical analysis for the prediction of cis-acting elements for each gene promoter was performed using the Plant-CARE online tool.

3.7. Interaction Network of GDSL Proteins

To further elucidate the function and regulatory network of the GDSL gene family, we analyzed and predicted the protein–protein interaction network. As illustrated in the figure, the GDSL interactive network in *M. domestica* consists of six distinct modules (Figure 6). Notably, Module 1 contains the highest number of members within the protein–protein interaction network, whereas Module 5 comprises the fewest, with only four proteins.

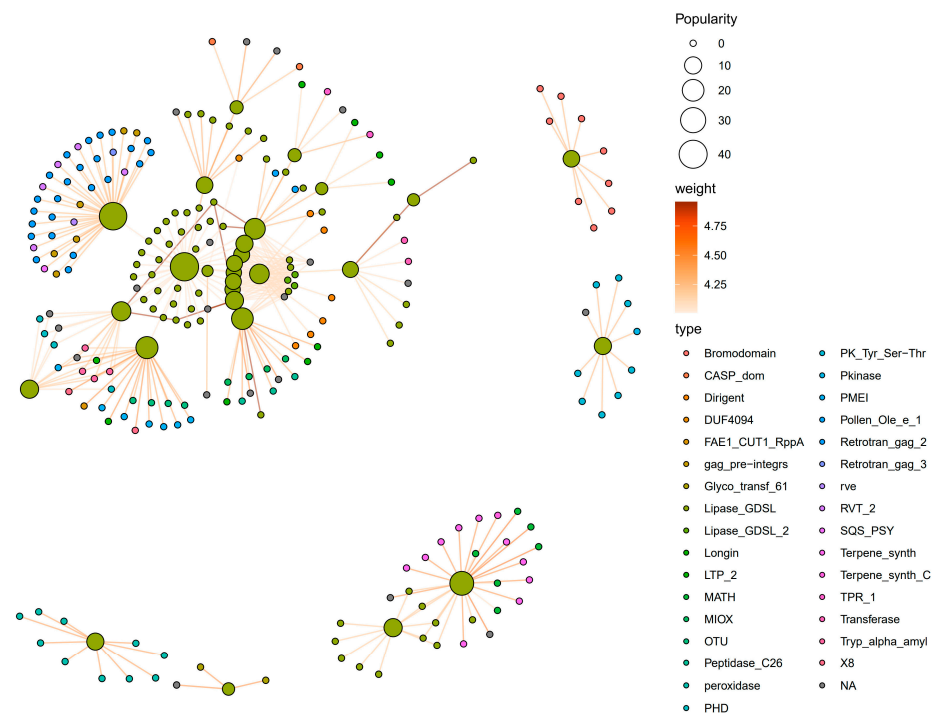


Figure 6. The interaction network between proteins for GDSL proteins in *M. domestica* is depicted. Various colors indicate distinct modules within the network.

Notably, significant protein–protein interactions were also identified among members of the GDSL protein family itself. These intrafamily interactions suggest their importance in maintaining functional synergy within the family and provide new insights into the specificity and redundancy of GDSL family proteins in particular biological pathways or cellular functions.

Importantly, GDSL proteins are capable of interacting not only with one another but also with other proteins, including Casparian strip membrane domain proteins (CASP) and Dirigent proteins. CASP and Dirigent proteins are known to play a crucial role in the development of casparian strip [36,37], suggesting that GDSL, CASP and Dirigent may synergistically contribute to this developmental process in *M. domestica*. This finding is sufficiently intriguing to merit further investigation.

3.8. Enrichment Analysis of the MdGDSL Genes

To further investigate the biological processes associated with MdGDSL genes, we conducted KEGG and GO analyses on 131 MdGDSL genes. The KEGG analysis revealed that these genes are involved in the biosynthesis and glycan degradation pathways of indole alkaloids in plants (Figure 7). We utilized Gene Ontology (GO) to classify the functional groups of these 131 genes, categorizing the MdGDSL genes into three GO classes: biological processes, cellular components, and molecular functions. Enrichment analysis was performed across these 3 categories, encompassing 19 biological process categories, 3 cellular component categories, and 6 molecular function categories. In the biological process category, the genes were primarily associated with plant hormones and responses to both biotic and abiotic stresses, including response to jasmonic acid, jasmonic acid and ethylene-dependent systemic resistance, response to wounding, and positive regulation of defense response. In the cellular component and molecular function categories, the focus was predominantly on the formation of the plant cell wall. The results indicated that GDSL may play a role in *M. domestica* growth.

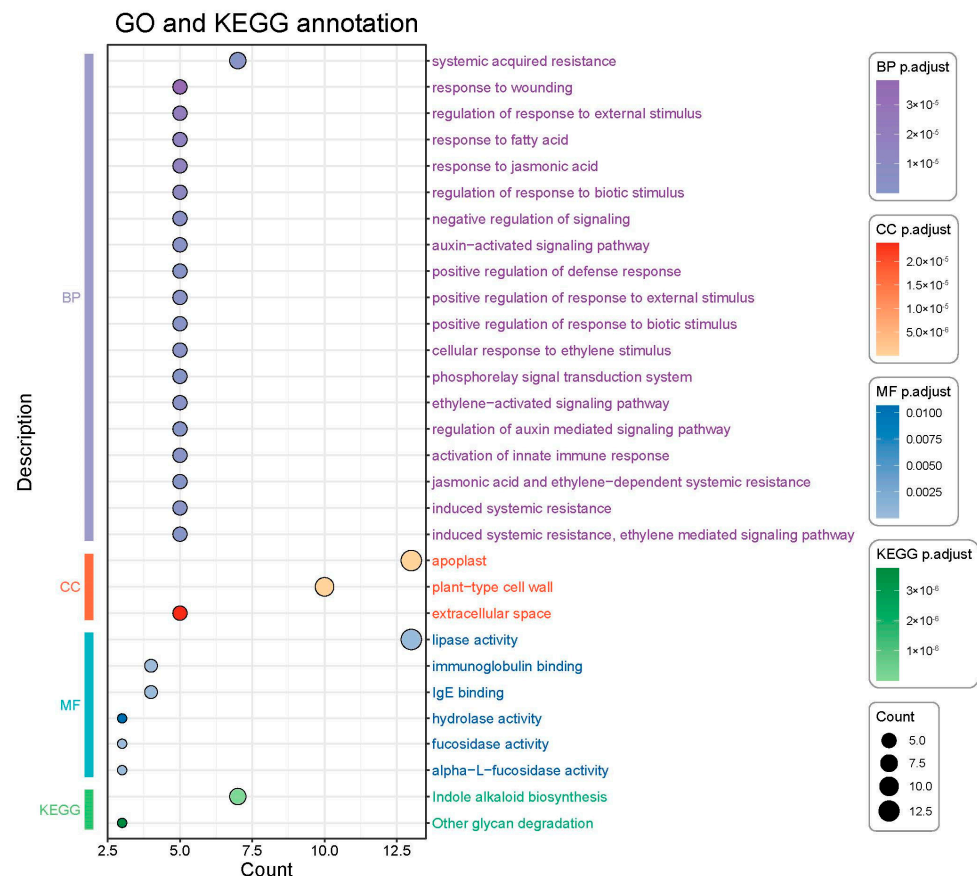


Figure 7. Functional annotations of GO and KEGG for GDSL genes in *M. domestica* were analyzed. The GO terms were categorized into three groups: biological process (BP), cellular components (CCs), and molecular function (MF). The vertical axis represents the annotated GO terms, while the horizontal axis displays the number of genes associated with these GO terms. BP, CC, and MF are represented by purple, red, and blue colors, respectively, whereas KEGG is indicated in green.

3.9. Expression Pattern Analysis of MdGDSL Genes Under Abiotic Stress by RNA-Seq

Previous studies have demonstrated that GDSL plays a crucial role in plant responses to abiotic stress (Ding et al., 2020; Aiello et al., 2020; Chiconato et al., 2021 [6–8]). In this study, we aimed to explore the potential biological function of the MdGDSL gene in response to abiotic stress by comprehensively analyzing the expression profile changes in the MdGDSL gene under high concentrations of CaCl_2 and cold stress using RNA-seq data. Based on previous studies, we established a differential expression threshold (up-regulated greater than 1.5, down-regulated less than 0.5) to identify genes that respond to stress (Li et al., 2015) [38]. As shown in Figure 8, our analysis indicates that, under high concentrations of CaCl_2 stress, the expression levels of Md10G1044500, Md13G1098900, Md12G1022800, Md16G1152900, Md09G1021900, Md09G1174700, Md15G1241800, Md11G1058600, Md11G1241800, Md05G1293600, Md15G1009100, Md05G1303900, Md10G1171100, Md09G1116600, Md02G1127000 and Md15G1009500 were significantly up-regulated (Figure 8). Additionally, we observed that the expression levels of certain MdGDSL genes decreased with prolonged exposure to high concentrations of CaCl_2 , while the expression levels of some MdGDSL genes increased over time. These findings suggest that MdGDSL gene expression is dynamically regulated in *M. domestica* subjected to high concentrations of CaCl_2 over extended periods.

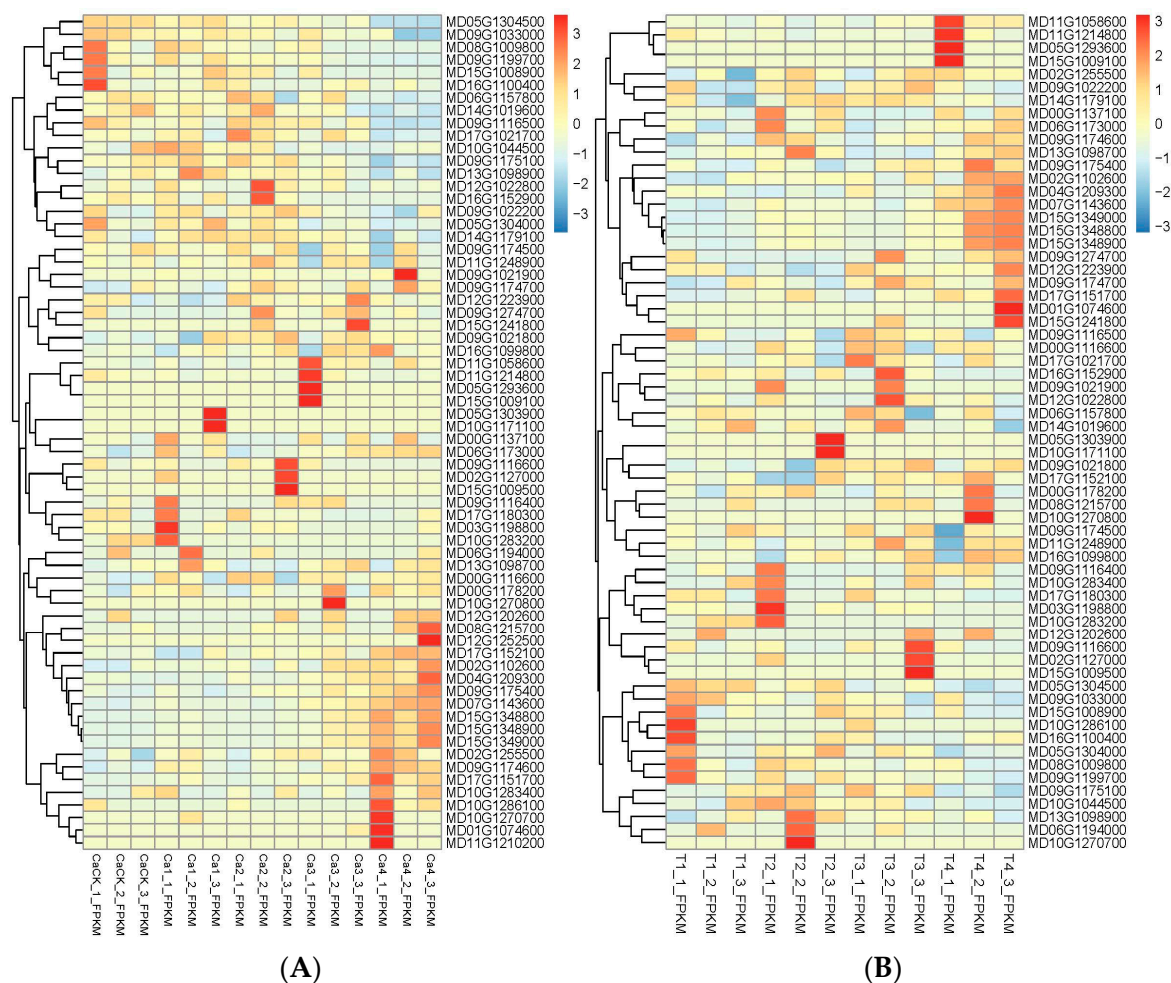


Figure 8. The expression profiles of GDSL genes in response to different levels of CaCl₂ and cold stress treatments are depicted. **(A)** The heatmap shows the relative expression ratios of MdGDSL genes in response to high concentrations of CaCl₂. **(B)** The heatmap presents the expression ratios of MdGDSL genes during low-temperature treatment. These data are visualized in a heatmap format, where gene expression is represented as row-scaled FPKM values across various treatments. High and low expression levels of MdGDSL genes are indicated by red and blue boxes, respectively.

After the cold treatment, the expressions of Md11G1058600, Md11G1214800, Md05G1293600, Md15G1009100, Md09G1175400, Md02G1102600, Md04G1209300, Md07G1143600, Md15G1349000 and Md15G1348800 genes were significantly up-regulated. The results indicate that MdGDSL genes may play a crucial role in the response of *M. domestica* to high CaCl₂ stress and low-temperature conditions. This analysis suggests that the expression patterns of MdGDSL genes may be complex and diverse under abiotic stress. Further research is necessary to elucidate the precise role of MdGDSL genes in stress response and their potential contribution to the adaptation and survival of *M. domestica* under stress.

3.10. Expression Pattern Analysis of MdGDSL Genes Under Cold Treatments by RT-qPCR

To further explore the potential function of MdGDSL genes, we selected five MdGDSL genes that exhibited responsiveness to cold stress conditions. We quantitatively analyzed their differential expression patterns using RT-qPCR after cold treatment (Figure 9). Notably, MD04G1209300, MD13G1098700, MD15G1348800, MD15G1349000 and MD15G1348900 exhibited upregulation in low temperature treatment without spray water (T2) and 20 mmol/L calcium chloride was sprayed and treated at a low temperature

(T4). Among them, *MD04G1209300*, *MD15G1348800* and *MD15G1349000* are significant on T4 (Figure 9). In contrast, under low-temperature treatment with spray water (T3), the expression of *MD04G1209300* and *MD15G1349000* increased. This discovery highlights the intricate and particular nature of the stress-responsive mechanisms associated with *MdGDSL* genes, indicating that these genes might have essential but distinct functions in facilitating plant adjustment to cold stress. In contrast, under low-temperature treatment with spray water (T3), the expression of *MD04G1209300* and *MD15G1349000* increased. This discovery highlights the intricate and particular nature of the stress-responsive mechanisms associated with *MdGDSL* genes, indicating that these genes might have essential but distinct functions in facilitating plant adjustment to cold stress.

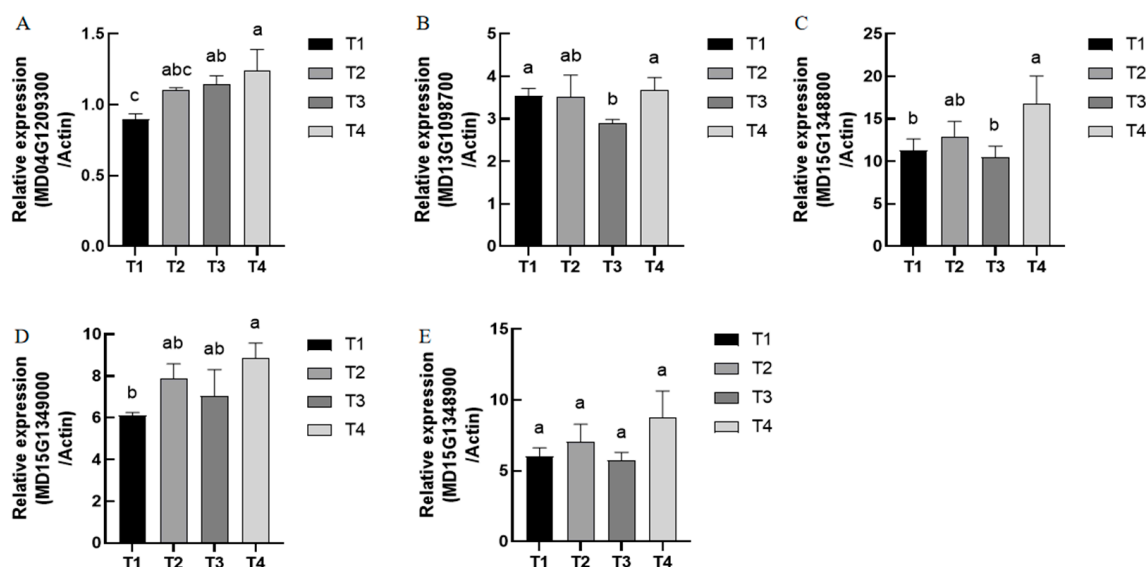


Figure 9. Expression patterns of *MdGDSL* genes under cold stress by RT-qPCR. (A–E) The expression level of *MD04G1209300* (A), *MD13G1098700* (B), *MD15G1348800* (C), *MD15G1349000* (D) and *MD15G1348900* (E) genes under cold treatment. The expression levels were calculated using $2^{-\Delta\Delta C_t}$ methods. Means $\pm$ SDs ($n = 6$). Different lowercase letters indicate significant differences among means ($p < 0.01$). qRT-PCR analysis were performed using the *MdActin* (*MD07G1012400*) as an internal control. (T1) flowers that were not subjected to low-temperature stress, (T2) flowers that experienced low-temperature stress treatment, (T3) flowers that experienced low-temperature stress treatment after water spraying, and (T4) flowers that were subjected to low-temperature stress treatment after spraying with 20 mmol/L calcium chloride.

4. Discussion

GDSL lipase and its hydrolysates play an important role in plant growth and development, as well as in biological and abiotic stress responses [4–9]. In recent years, the diverse biological functions of GDSL lipase have attracted increasing attention from plant scientists. Research has demonstrated that the number of GDSL lipase genes varies across different plant species. Notably, *M. domestica* possess a substantial number of GDSL lipase genes (up to 131) distributed irregularly across various chromosomes. In comparison to *A. thaliana*, *Z. mays* and *O. sativa*, *M. domestica* exhibit a higher number of GDSL lipase genes; however, this number is still lower than that found in *G. max*, *D. villosum*, *G. hirsutum*, *G. barbadense*, *G. tomentosum*, *G. mustelinum* and *G. darwinii* [12,22–25,39], indicating significant variation among species. The GDSL lipase gene is also present in *Penium margaritaceum*, which is believed to be the last common ancestor of the two star green algae and plants [40]. *Selaginella moellendorffii*, an ancient plant, underwent significant GDSL lipase gene expansion shortly after the transition of plants to terrestrial environments [40]. Shen et al. proposed that the GDSL lipase gene plays a pivotal role in the evolutionary transition of

plants from aquatic to terrestrial habitats [41]. Consequently, the GDSL lipase gene is found in all terrestrial plants. Based on these findings, we hypothesize that the abundance of GDSL lipase genes is related to their unique functional roles within different species.

Protein structure prediction has revealed a high degree of structural diversity among members of the GDSL lipase family. Furthermore, significant variations in the basic physicochemical properties of 131 GDSL lipases were observed, indicating substantial genetic diversity within the *M. domestica* GDSL lipase family. Chromosomal localization and collinear analysis results demonstrated an irregular distribution of MdGDSL, with most members located in repeated sequences across various chromosomes, as well as in clusters and tandem arrangements. Previous research has shown that 6 GDSL lipase genes (EXL1-6) in *A. thaliana* are arranged in tandem near the centromere of the long arm of chromosome 1 [42], while 54 OsGDSL genes in *O. sativa*, constituting 47% of the total, are densely clustered at 17 chromosomal loci [22]. Further investigation revealed that WGD significantly contributed to the expansion of the MdGDSL gene family, whereas the expansion of the GDSL gene family in *A. thaliana* and *V. vinifera* was primarily driven by TD. These observations suggest that GDSL genes are functionally conserved throughout evolution, yet exhibit considerable differentiation among different species. Such findings have important implications for understanding how plants adapt to complex and dynamic environments.

Gene structure analyses reveal that members of the MdGDSL family typically possess four to five exons, while the structure and length of their introns exhibit considerable variability. The processes of intron deletion and addition serve as significant driving forces behind their functional diversification [43]. This suggests that MdGDSL members have evolved distinct functions to adapt to various physiological processes, including plant growth and development, organ morphogenesis, and stress responses. Predictions regarding cis-regulatory elements further substantiate this conclusion. The promoter of the GDSL lipase gene contains a substantial number of cis-acting elements associated with plant growth and development, hormonal regulation, and responses to both biotic and abiotic stresses. Certain genes are notably induced under cold conditions and high concentrations of calcium chloride stress. Previous studies have also indicated that GDSL lipase plays a critical role in various physiological and biochemical processes. These findings suggest that GDSL lipase may play a role in the growth, development and stress response of *M. domestica*.

5. Conclusions

In this study, we identified a total of 131 GDSL genes in the *M. domestica* genome. Phylogenetic analysis indicates that GDSL can be categorized into four subfamilies. Gene duplication events suggest that WGD is a significant driver of the expansion of the MdGDSL gene family. Genes within the same subgroup exhibited similar conserved motifs and gene structures. Collinearity analysis revealed a greater number of collinear gene pairs between *M. domestica* and *V. vinifera* compared to those between *M. domestica* and *A. thaliana*. Protein interaction predictions indicated that MdGDSL proteins can form heterodimers and interact with proteins associated with the development of the Casparian strip, suggesting that the MdGDSL gene may play a crucial role in this developmental process. GO annotation showed that the MdGDSL gene is primarily involved in stress response mechanisms. Furthermore, we found that the MdGDSL gene may significantly contribute to the response of *M. domestica* to high concentrations of CaCl₂ and cold stresses. This study establishes a foundation for further investigation into the function of the MdGDSL gene in *M. domestica* and other plant species.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy15030517/s1>. Supplemental Data S1. Analysis of physicochemical properties of GDSL gene family in *M. domestica*. Supplemental Data S2. Ka/Ks and Ka/Ks values of GDSL duplicate gene pairs in *M. domestica* and *V. vinifera* and *A. thaliana*. Supplemental Data S3. Ka, Ks and Ka/Ks values of syntenic gene pairs between *M. domestica* and *V. vinifera* and *A. thaliana*. Supplemental Data S4. The primer sequences of MDGDSL genes used for RT-qPCR.

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