Original Research

Comprehensive Characterization and Transcriptomic Profiling of the *GH3* Gene Family in *Brassica rapa* and Its Involvement in Auxin-Mediated Growth and Stress Response
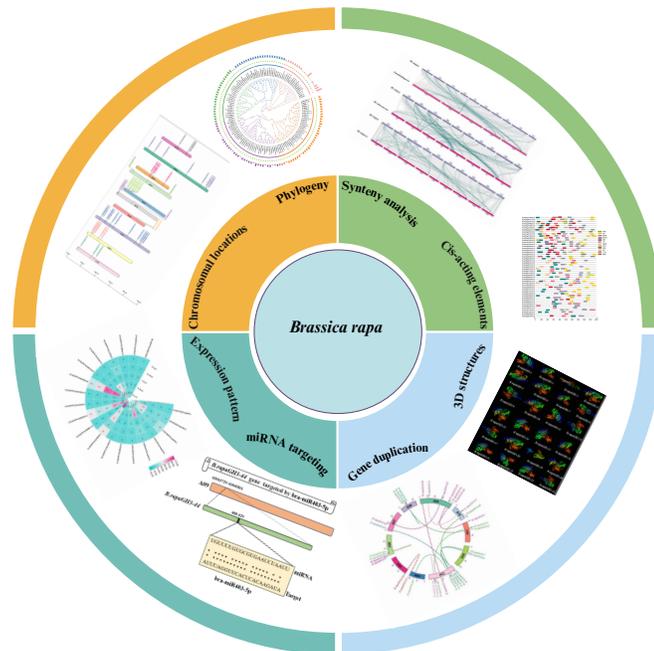
Javaria Tabusam*, Yixing Zhang, Yi Ren

Highlights

- Genome-wide identification and classification of 49 *GH3* genes in *Brassica rapa*
- Integrated structural, regulatory, and expression analyses provide functional insights into *GH3* genes.
- Transcriptomic and promoter analyses link *GH3* genes to hormone signaling and stress responses

Graphical Abstract

Abstract

The Gretchen Hagen 3 (*GH3*) gene family plays a central role in maintaining auxin homeostasis and regulating hormone-mediated growth and stress responses in plants. In this study, a comprehensive genome-wide analysis of the *GH3* gene family in *Brassica rapa* identified 49 *GH3* genes. Phylogenetic analysis classified these genes into five distinct groups, reflecting their evolutionary relationships. Gene structure and conserved motif analyses revealed considerable variation in exon–intron organization among *GH3* genes, while motif composition remained highly conserved within each phylogenetic group, suggesting functional conservation. Promoter analysis identified numerous *cis*-acting regulatory elements associated with phytohormone responsiveness, including auxin, abscisic acid, salicylic acid, gibberellins, and jasmonates, as well as elements related to abiotic stresses such as drought, low temperature, light, and defense responses. In addition, multiple microRNAs were predicted to target *B. rapaGH3* genes, indicating an additional layer of post-transcriptional regulation. Transcriptomic analysis further revealed distinct tissue-specific and developmental expression patterns of *GH3* genes, highlighting their diverse regulatory roles during plant growth. Overall, this study provides a systematic framework for understanding the structural, regulatory, and expression characteristics of the *GH3* gene family in *B. rapa* and offers valuable insights for future functional studies aimed at improving stress tolerance and developmental traits in Brassica crops.

1. Introduction

Auxin is a key phytohormone regulating plant growth, development, and stress responses through cell expansion, division, and gene regulation (Hoffmann et al., 2011; Zhao, 2010). Auxin-responsive gene families known as indole acetic acid/ auxin (IAA/Aux), small auxin up RNA (SAUR), and Gretchen Hagen 3 (*GH3*) are mainly involved in these processes to regulate plant growth and resistance to stresses (Abel and Theologis, 1996). These gene families play a significant role in maintaining auxin homeostasis through an effective mechanism, such as IAA, which is associated with amino acid to inactivate this with the help of the GH3 family (Fu et al., 2011; Chapman and Estelle, 2009). The *GH3* gene first identified in *Glycine max* (Hagen et al., 1984; Takase et al., 2003), has since been characterized across diverse species, including *Arabidopsis thaliana* (Park et al., 2007), *Malus domestica*

(Yuan et al., 2013), *Solanum lycopersicum* (Kumar et al., 2012), *Zea mays* (Feng et al., 2015), *Citrus sinensis* (Xie et al., 2015), *Oryza sativa* (Jain et al., 2007), and *Bryophytes* (Ludwig-Müller et al., 2009).

GH3 plays a crucial role in the growth and development, as well as stress tolerance in plants against biotic and abiotic stress. However, gene expression of *GH3* genes is regulated by many environmental factors and phytohormonal activity (Böttcher et al., 2010). Several *cis*-acting elements involved in transcriptional activation, such as AuxRE and an additional assortment of hormone- and stress-related elements, have also been identified in the promoter region of the *GH3* gene (Ostrowski and Jakubowska, 2013; Woodward and Bartel, 2005). Considering the substrate specificity and sequence similarity, members of the *GH3* family are distinguished into three groups (group I, group II, and group III) (Staswick and Tiryaki, 2004). Based on the roles of GH3 proteins, they are categorized into three main groups. Group I plays a role in JA- and SA-amido synthetase. Group II *GH3* proteins are involved in IAA-amido synthetase activity, which in turn regulates auxin homeostasis, while Group III GH3 proteins have only been identified in *Arabidopsis* thaliana (Staswick et al., 2002). At*GH3*.5 is indirectly involved in hypocotyl growth by interacting with the activity of IAA-amido synthase (Park et al., 2007). In another experiment, they found that in an *Arabidopsis GH3*.9 mutant (*GH3*.9-1), it could grow a longer primary root than the wild type. This gene is also associated with the signaling pathway of jasmonate and auxin crosstalk (Khan and Stone, 2007a). The At*GH3*a/*GH3*.5 gene regulates the expression of the PR-1 gene, which, in turn, is responsible for plant responses to stress. Jagadeeswaran et al. (2007) reported that the *Arabidopsis* mutant with the knocked-out *GH3*.12 gene was highly susceptible to the bacterium *Pseudomonas syringae* in comparison to the wild type of plant. Furthermore, in *Capsicum chinense* L., *GH3* family members (Cc*GH3*) induce fruit ripening that is regulated by auxin and ethylene (Liu et al., 2005). The role of the *GH3* family still needs to be explored in several economically important and notorious agricultural and horticultural plants, such as the *Brassica* family. The Os*GH3*.8 gene in *Oryza sativa* is responsible for resistance triggered against Xanthomonas oryzae. The overexpression of this gene is not only correlated with disease resistance but also inhibits cell expansion by suppressing genes involved in this pathway, ultimately leading to delayed plant growth and development (Ding et al., 2008). While the overexpression of Os*GH3*.5 and Os*GH3*.13 is associated with the bending of the leaf blades (*lamina inclination*) and changes in root morphology, respectively (Domingo et al., 2009, Zhang et al., 2015, Du et al., 2012, Zhang et al., 2009). Studies show that *Colletotrichum graminicolum* infection in Zea mays causes the upregulation of Zm*GH3*-2 and Zm*GH3*-8 genes, suggesting that these two genes play an important role in response to this disease attack (Kong et al., 2019). In Solanum tuberosum, St*GH3*.1 and St*GH3*.5 genes knockout or downregulation causes reduced resistance against *Ralstonia solanacearum* (Zou et al., 2022). *Brassica rapa*, an economically important leafy vegetable crop (2n = 20, AA genome), exhibits diverse leaf morphologies influenced by auxin signaling (Cartea et al., 2010; Chen et al., 2011). Despite extensive *GH3* studies in model species, no comprehensive genome-wide analysis exists for *B. rapa*. Here, we identified 49 *B. rapaGH3* genes and conducted phylogenetic, structural, *cis*-regulatory, miRNA, and transcriptomic analyses to elucidate their roles in auxin-mediated growth and stress responses.

Brassica is one of the most important genera in the Brassicaceae family, exhibiting economically important crops like mustard, cabbage, rape, and cauliflower (Cartea et al., 2010). *Brassica rapa* (*B. rapa*) is a plant species that originally belonged to the Cruciferae (Brassicaceae) family and is divided into 3 categories: *B. rapa* oil type, *B. rapa* leafy type, and *B. rapa* rapiferous type (Miceli et al., 2019). *B. rapa* (2n = 20, AA) is one of the six economically important Brassica species produced in the U's triangle (Chen et al., 2011). All of these leafy vegetables are plant leaves that are consumed as vegetables; leaves are secondary crops whose primary purpose is photosynthesis and food production. *B. rapa* has attained significance due to the variety of leaf shapes, such as leaf curvature and heading trait, which are widely debated due to their mineral accumulation and nutrient availability characteristics. The *GH3* family plays a vital role in the growth and establishment of different leaf

morphologies in *B. rapa*. An auxin pathway gene (Br*GH3*.12) was reported in overlapping and curling Chinese cabbage leaves (Gu et al., 2017). Any mutation in these genes could produce abnormal or different leaf forms in vegetables (Odhav et al., 2007, Liu et al., 2010).

Genome-wide studies enable researchers to characterize the gene families, structures, evolutionary expression patterns, protein interactions, etc., by accessing online public data (Safder et al., 2022). Nevertheless, no genome-wide study of the *GH3* gene family has been conducted in *B. rapa*. In this study, we used in silico approaches to identify 49 *GH3* genes in *B. rapa*, construct phylogenetic trees and synteny relationships, predict promoter *cis*-regulatory elements and protein structures, and perform transcriptomic profiling using public RNA-seq datasets. These analyses reveal expression patterns across developmental stages and tissue types, providing comprehensive insights into the structure, evolution, and function of the *GH3* gene family.

2. Materials and Methods

2.1. Identification and Characterization of the *GH3* Gene Family in *Brassica rapa*

To identify the *GH3* gene family members in the target species, the amino acid sequences of *A. thaliana GH3* genes were retrieved from the *Arabidopsis* genome database (TAIR) (https://www.arabidopsis.org/). *Arabidopsis* genes were used as query sequences in BLASTP searches against the Brassica database (http://brassicadb.cn/#/BLASTP/) to identify *B. rapaGH3* genes. Additional BLASTP searches against multiple *Brassica* species used a less stringent e-value of 1e-5 to detect divergent homologs potentially masked by high stringency. *GH3* domain profiles (PF03321.16) were obtained from Pfam (http://pfam.xfam.org/) and searched using HMMER 3.1 (http://www.hmmer.org/) with default settings. Forty-nine *B. rapaGH3* genes were validated using Ensembl Plants (https://plants.ensembl.org/Brassica_rapa/Info/Index), characterized for physicochemical properties (ProtParam: https://web.expasy.org/protparam/), subcellular localization (Wolf PSORT: https://wolfpsort.hgc.jp/; TargetP), conserved domains (NCBI CDD) (Gasteiger et al., 2005), motifs (MEME Suite), intron-exon structure (GSDS 2.0: http://gsds.cbi.pku.edu.cn/), and protein interactions (STRING: http://string-db.org/).

2.2. Phylogeny and Synteny Analysis

GH3 protein sequences from *Arabidopsis thaliana* and *Brassica* species (*B. juncea, B. oleracea, B. nigra, B. napus, B. carinata*) were retrieved by BLASTP and aligned using MEGA X. Phylogenetic trees were constructed using Neighbor-Joining (NJ; 1000 bootstrap replicates) and Maximum Likelihood (ML; JTT model) methods, with bootstrap values >70 indicating strong support (Tamura et al. 2021). Synteny relationships were analyzed using MCScanX and visualized with TBtools dual synteny plots (https://github.com/CJ-Chen/TBtools) and Circos circular plots.

2.3. Gene Duplications and Purity Selection

MCScanX and Advanced Circos features were used to identify duplicated genes (Wang et al. 2012). A custom Python script was developed to automate the extraction of gene pairs for tandem duplication events, ensuring accurate categorization. TMY was estimated based on prior research in *B. rapa* (Mun et al. 2009), and the Ka/Ks ratios were computed using the Ka/Ks calculator in TBtools. Values <1 indicate purifying selection, supporting evolutionary conservation of the *B. rapaGH3* gene family (Guéguen and Duret, 2018). TBtools was used to perform a synteny analysis for the prediction of correlations between *B. rapa* and its closely related species (Chen et al. 2018). To account for variations in evolutionary rates, additional analyses were conducted using the PAML (Phylogenetic Analysis by Maximum Likelihood) package.

2.4. *Cis*-element and Protein Structure of GH3 in *B. rapa*

Promoter sequences (2000 bp upstream of the start codon) of *B. rapaGH3*

genes were retrieved from the BRAD database (http://brassicadb.cn) for *cis*-regulatory element analysis. The web-based PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was utilized for further categorization. Using the fold recognition approach and the web-based program PHYRE2 (PHYRE2 Protein Fold Recognition Server; ic.ac.uk), the three-dimensional (3D) structure of the *B. rapaGH3* genes was visualized. Furthermore, molecular docking studies to analyze the binding of the obtained molecules to the targets were performed by the Auto Dock Vina tool to identify the GH3 proteins and their substrates, such as auxins and other signaling molecules.

2.5. Targeted miRNA prediction and functional analysis

The psRNATarget database (http://plantgrn.noble.org/psRNATarget) was used to determine gene interactions with microRNAs, and the interactions were further predicted by graphical illustration. To increase the accuracy of target prediction, the criteria for target pairing were adjusted to allow a maximum mismatch score of 3, ensuring that only high-confidence interactions were included. OmicShare (https://www.omicshare.com/) and KEGG analysis (http://brassicadb.cn/#/Annotations/) were used to determine the functional pathway of *B. rapaGH3*.

2.6. Expression Analysis of *B. rapaGH3*

Using the "Transcriptomic" tool from BRAD (http://brassicadb.cn/#/Transcriptome/), TbTools was used to create a heat map using RNA-seq data to ascertain the expression of *GH3* genes in various *B. rapa* tissues. The data used for the expression profiling were retrieved from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE106444. Furthermore, the transcriptomic analysis of *B. rapaGH3* orthologs in *Arabidopsis* was also retrieved from (https://www.ncbi.nlm.nih.gov/sra/SRX1994959).

3. Results

3.1. Identification and Characteristics of *GH3* Gene Family Members in *B. rapa*

Using *Arabidopsis thaliana* GH3 protein sequences as BLASTP queries, 49 *GH3* genes were identified across all 10 *B. rapa* chromosomes (A01-A10) and designated *B. rapaGH3*-1 through *B. rapaGH3*-49, each containing the canonical *GH3* domain (PF03321.16) essential for auxin-amido synthetase activity. Phylogenetic analysis resolved these genes into five distinct clades with strong bootstrap support (>70%), where genes within each clade exhibited identical motif compositions indicative of conserved enzymatic functions (Fig. 1A). Eight conserved motifs were characterized, including motif 1 (WEGIITRLWPNTKYI), motif 2 (PBEVILCPDSKQSMY), and motif 8 (KPQDVSYTFLPNMS) with group-specific distributions suggesting functional specialization. The 49 *B. rapaGH3* genes displayed substantial physicochemical diversity, with lengths ranging from 119 aa (*B. rapaGH3*-27) to 881 aa (*B. rapaGH3*-13), molecular weights from 13.4-100.1 kDa, and pI values spanning 4.86-7.81 (Table. 1). Subcellular localization predictions revealed distinct compartmentalization patterns: 26 proteins (53%) localized to the plasma membrane, 18 (37%) to the nucleus, 4 (8%) to the cytoplasm, and 1 (2%) extracellularly, suggesting diverse roles in auxin transport, transcriptional regulation, metabolic processing, and intercellular signaling (Fig. S1).

Variations in exon–intron organization may reflect differential regulatory complexity (Fig S2). Intron-exon structures varied widely from 1 exon (*B. rapaGH3*-26) to 14 exons (*B. rapaGH3*-13), with most genes containing 3-6 exons (Fig S2). Nuclear-localized genes exhibited more complex architectures with higher exon numbers, while cytoplasmic genes typically had fewer introns, potentially indicating differences in regulatory complexity and expression timing. The diversity among exon-intron structures indicates that the *B. rapaGH3* gene family has undergone distinct adaptive evolution to play broad roles in auxin signaling, stress, and growth regulation. These findings suggest that these genes have diverse roles, where some require a fast response

to stimuli while others are most probably involved in developmental gene regulation.

3.2. Chromosomal Distribution of *B. rapaGH3* Genes

All 10 *B. rapa* chromosomes (A01-A10) contained *GH3* genes, confirming genome-wide distribution of this auxin-responsive family. Chromosome A03 harbored the highest density with 11 genes (22% of total), establishing it as the primary *GH3* hotspot. Chromosomes A06 and A09 each contained 8 genes (16% each), while chromosome A05 had the fewest with only 2 genes (4%). Chromosomes A01 (5 genes), A02, A04, A07, and A10 (3-4 genes each) showed intermediate densities (Fig. 2 and Fig. S3). Chromosome A03 exhibited distinct gene clustering between 20-40 Mb, with multiple *B. rapaGH3* loci in proximity, characteristic of tandem duplication events. This regional concentration suggests local gene expansion through unequal crossing-over or replication slippage. Chromosome A06 showed a more dispersed distribution across its length, with *GH3* genes separated by larger intergenic regions, indicating segmental duplication origins rather than tandem arrays. Notably, genes within individual phylogenetic clades maintained consistent molecular features across chromosomes: identical motif compositions, similar exon-intron architectures, and matching subcellular localizations. This cross-chromosomal conservation within clades demonstrates that *B. rapaGH3* diversification occurred post-duplication rather than through independent chromosomal evolution.

3.2. Phylogeny and Evolutionary Relationship of *B.rapaGH3* Gene

Comparative phylogenetic analysis included *B. rapaGH3* genes alongside *GH3* family members from *Arabidopsis thaliana* (*AtGH3*), *B. nigra* (*B. nigGH3*), *B. oleracea* (*B. oleGH3*), *B. juncea* (*B. juGH3*), *B. napus* (*B. napGH3*), and *B. carinata* (*B. carGH3*). The phylogenetic tree was divided into five groups named as Group 1, Group 2, Group 3, Group 4, and Group 5 (Fig 3). Group 1 consisted of 16 *GH3* members (6 *AtGH3, 5 B.rapaGH3, 3 B.oleGH3, 1 B.nigGH3,* and *1 B.JuGH3*); Group 2 consisted 22 members (96 At*GH3* and 13 *B.rapaGH3*), Group 3 consisted 25 members (1 *AtGH3*, 6 *B.rapaGH3*, 3 *B.oleGH3*, 7 *B.napusGH3*, 2 *B.nigGH3*, and 6 *B.JuGH3*), Group 4 consisted 35 members (4 *AtGH3*, 18 *B.rapaGH3*, 2 *B.oleGH3*, 1 *B.napusGH3*, 3 *B.nigGH3*, and 7 *B.cariGH3*) and Group 5 consisted 36 members (2 At*GH3*, 7 *B.rapaGH3*, 4 *B.oleGH3*, 8 *B.napusGH3*, 3 B.nig*GH3*, 6 B.cari*GH3*, and 6 *B. Juncea*). All these groups indicated a close relationship within the *GH3* gene family and strongly supported the conclusion of a common originator (Song et al. 1988). This interspecies phylogeny confirms that *B. rapaGH3* diversification reflects both ancient duplication (clade formation) and recent expansion (chromosomal hotspots), establishing conserved functional modules across Brassicaceae.

3.3. *GH3* Gene Duplication and Ka/Ks Analysis

Gene duplication and selection pressure analysis of the 49 identified *B. rapaGH3* genes, 15 (31%) participated in duplication events, including 5 segmental and 10 tandem duplication pairs (Fig. 4A; Table. 2). Tandem duplications, comprising 67% of duplicate pairs, were predominantly clustered on chromosome A03 within the 20-40 Mb region previously identified as a *GH3* hotspot (Fig. 2), while segmental duplications spanned A03-A06 and A01-A09 pairs, consistent with *B. rapa* whole-genome triplication history (Magadum et al. 2013). WGD, also known as polyploidization, is an extreme form of gene duplication that results in an intrusive expansion in both genome size and gene set size. Many important developmental and regulatory genes in plants are the consequence of these duplication events. Gene duplication, in addition to facilitating critical developmental functions, also continued to play an eventual role in the evolutionary arms race between plants and pathogens/herbivores (Ishizaki 2017). These results suggest a strong evolutionary constraint on duplicated GH3 genes. Synonymous substitution rates (Ks) ranged from 0.08-0.62, corresponding to divergence times $T = Ks/(2\lambda)$ of 3.2-24.8 Mya using *B. rapa*-calibrated substitution rates ($\lambda = 1.5 \times 10^{-8}$ substitutions/site/year; Mun et al., 2009).
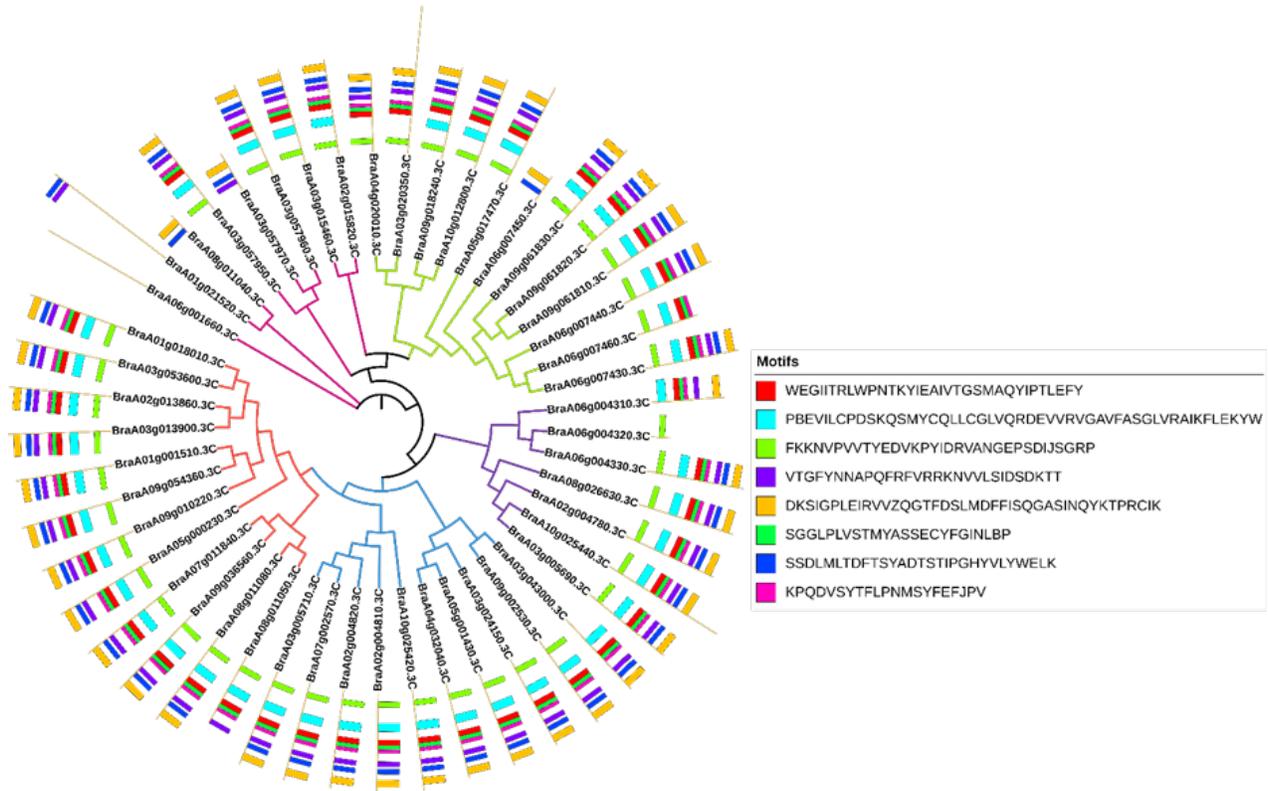
Fig. 1. Phylogenetic and graphical representations of the conserved motif in *B. rapaGH3*.
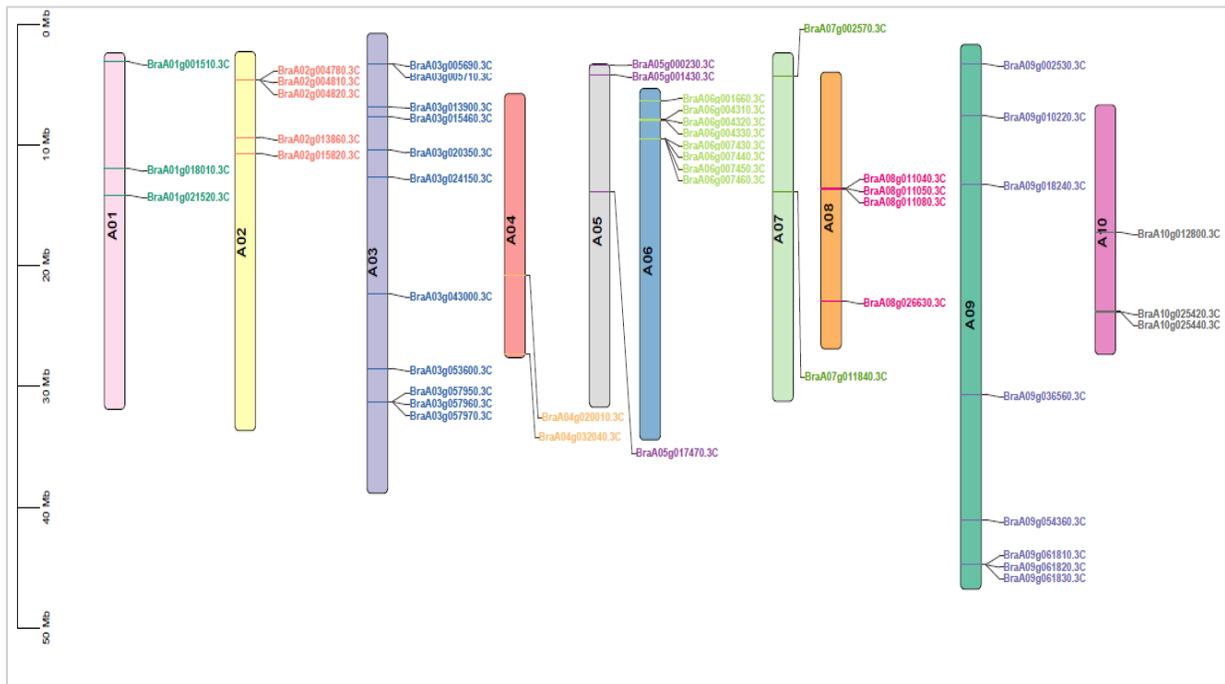

Fig. 2. The distribution of *GH3* gene family members across *B. rapa* chromosomes is shown in a chromosomal map. The size of each chromosome is indicated on the left scale.

Recent tandem duplicates on A03 (<5 Mya, Ks = 0.08-0.15) contrasted with older segmental duplicates (15-25 Mya), aligning with Brassica triplication timing. These duplication patterns correlate with prior observations: A03 tandem clusters account for 67% of tandem events, phylogenetic Group IV expansion (18 genes) contains 5/15 duplicate pairs, and plasma membrane-localized Group I/II genes exhibit the lowest Ka/Ks ratios (0.12-0.28),

indicating the strongest functional conservation. The Ka/Ks ratio indicates the type of selective pressure acting on genes. According to the results, purifying selection is the main determinant for the evolution of *B.rapaGH3* genes (Guéguen and Duret 2018). Divergence times TM Y were calculated with T = K s / 2x, using x values from other *B. Rapa* studies (Mun et al. 2009) (Table 2).
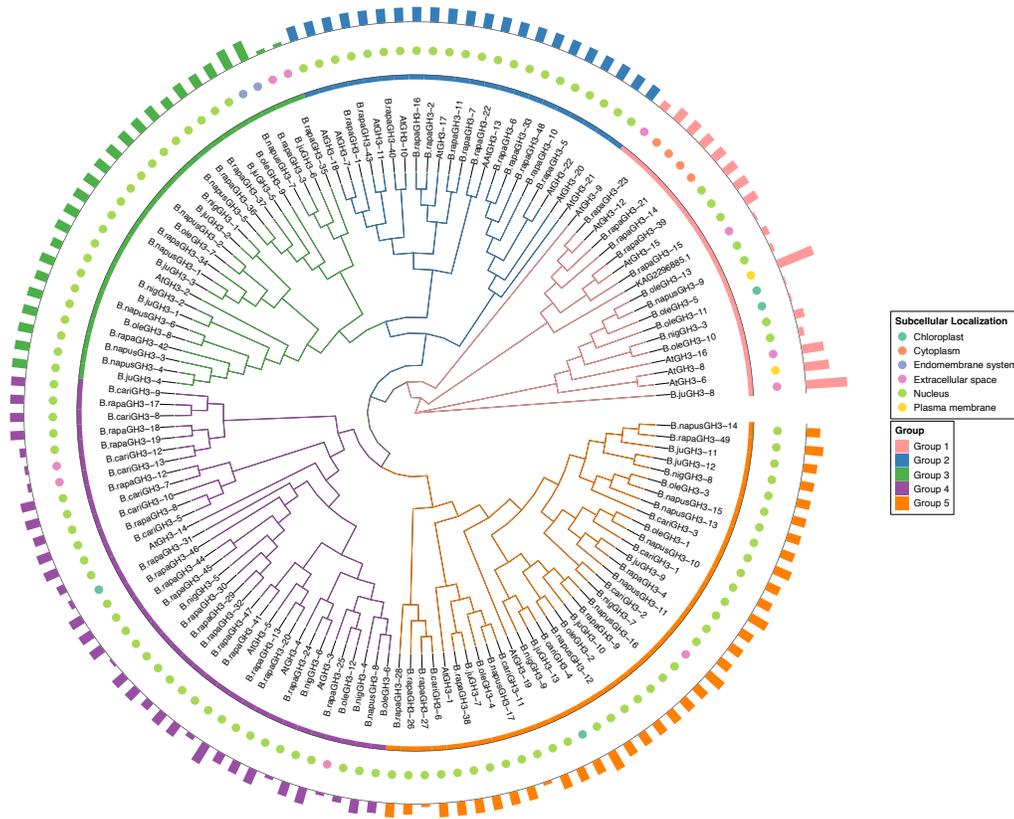
Fig. 3. Phylogeny of *B. rapaGH3* genes with *Brassica/Arabidopsis* orthologs. Five clades (I-V, color-coded). Outer ring: subcellular localization.
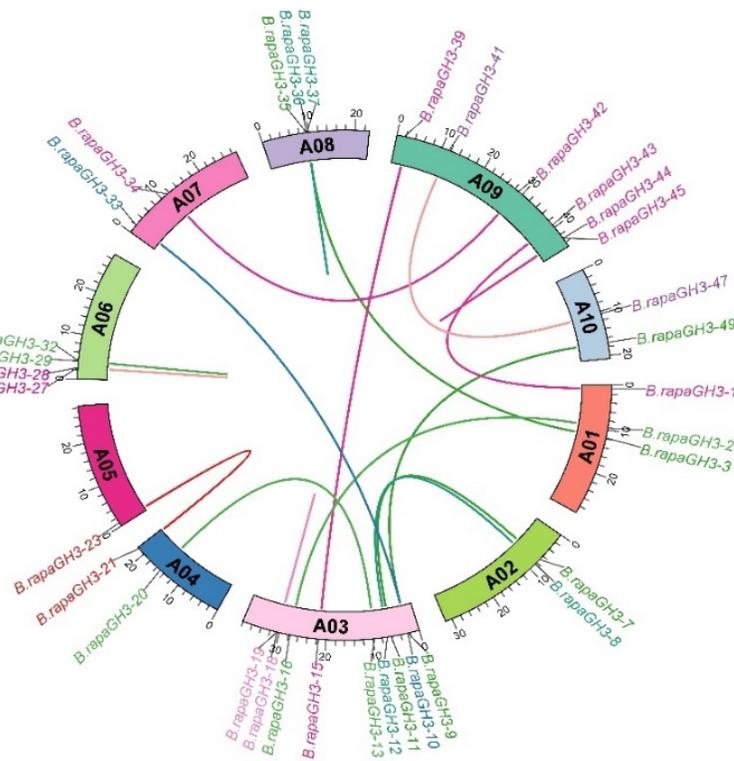

Fig. 4. Gene duplication of *B. rapaGH3* genes. The associations between duplicate pairs of *B. rapaGH3* genes are shown by the lines.

Table 1. Physical and Biochemical properties of *GH3* family members in *B. rapa*

Transcript ID	Gene Name	Chr Position	Start	End	Strand	Protein Length (A.A)	Protein MW (KDa)	Instability Index	PI	GRAVY	Intron-Exon	Sub-cellular Localization
BraA01g001510.3C	*B.rapaGH3-1*	A01	761309	763402	-	587	66034.44	48.31	5.98	-0.288		Nuclear
BraA01g018010.3C	*B.rapaGH3-2*	A01	9589366	9591863	+	612	69357.43	51.35	6.05	-0.29	3,2	Nuclear
BraA01g021520.3C	*B.rapaGH3-3*	A01	11790980	11795560	+	826	90766.13	51.73	7.33	-0.252	10,9	Plasma Membrane
BraA02g004780.3C	*B.rapaGH3-4*	A02	2321653	2323869	+	570	64765.54	39	5.14	-0.269	4,3	Nuclear
BraA02g004810.3C	*B.rapaGH3-5*	A02	2345906	2348749	+	595	67019.9	38.27	5.8	-0.118	5,4	Plasma Membrane
BraA02g004820.3C	*B.rapaGH3-6*	A02	2349848	2359986	+	590	66494.61	37.09	6.27	-0.133	6,5	Nuclear
BraA02g013860.3C	*B.rapaGH3-7*	A02	7064752	7066957	+	612	69094.92	48.57	5.87	-0.283	3,2	Nuclear
BraA02g015820.3C	*B.rapaGH3-8*	A02	8399328	8401338	+	577	65543.87	44.4	5.56	-0.163	4,3	Plasma Membrane
BraA03g005690.3C	*B.rapaGH3-9*	A03	2500376	2502398	+	573	65609.1	39.28	5.65	-0.258	4,3	Nuclear
BraA03g005710.3C	*B.rapaGH3-10*	A03	2512636	2515114	+	593	66391.99	38.44	5.38	-0.179	5,4	Cytoplasmic
BraA03g013900.3C	*B.rapaGH3-11*	A03	6101715	6103839	+	608	68626.48	49.72	5.74	-0.253	3,2	Nuclear
BraA03g015460.3C	*B.rapaGH3-12*	A03	6910366	6912368	-	580	66048.68	36.2	5.81	-0.152	4,3	Plasma Membrane
BraA03g020350.3C	*B.rapaGH3-13*	A03	9682251	9687894	-	881	100123.78	50.43	5.21	-0.333	14,13	Nuclear
BraA03g024150.3C	*B.rapaGH3-14*	A03	11886735	11888866	+	578	65129.54	45.75	5.65	-0.174	3,2	Plasma Membrane
BraA03g043000.3C	*B.rapaGH3-15*	A03	21597723	21599914	-	566	63462.48	38.87	5.65	-0.154	4,3	Plasma Membrane
BraA03g053600.3C	*B.rapaGH3-16*	A03	27739546	27741846	+	612	69388.58	47.47	5.98	-0.267	3,2	Nuclear
BraA03g057950.3C	*B.rapaGH3-17*	A03	30489205	30491488	-	572	64422.84	41.24	5.74	-0.155	4,3	Cytoplasmic
BraA03g057960.3C	*B.rapaGH3-18*	A03	30492428	30494690	-	582	65716.22	41.92	5.92	-0.181	4,3	Cytoplasmic
BraA03g057970.3C	*B.rapaGH3-19*	A03	30495644	30496488	-	252	28506.6	25.55	5.15	-0.034	2,1	Plasma Membrane
BraA04g020010.3C	*B.rapaGH3-20*	A04	15050830	15052729	+	464	52510.3	45.9	6.35	-0.178	5,4	Nuclear
BraA04g032040.3C	*B.rapaGH3-21*	A04	21599502	21601390	+	573	64465.84	41.12	5.92	-0.14	3,2	Plasma Membrane
BraA05g000230.3C	*B.rapaGH3-22*	A05	119629	122314	+	610	68947.55	42.06	5.63	-0.147	3,2	Nuclear
BraA05g001430.3C	*B.rapaGH3-23*	A05	879064	881435	-	579	65066.42	42.03	5.81	-0.18	4,3	Cytoplasmic
BraA05g017470.3C	*B.rapaGH3-24*	A05	10531830	10538007	-	635	71096.03	48.31	5.72	-0.146	5,4	Plasma Membrane
BraA06g001660.3C	*B.rapaGH3-25*	A06	1033285	1037237	-	671	75774.57	45.83	5.87	-0.712	12,11	Nuclear
BraA06g004310.3C	*B.rapaGH3-26*	A06	2576700	2578019	-	439	49834.76	36.52	4.94	-0.181	1,0	Plasma Membrane
BraA06g004320.3C	*B.rapaGH3-27*	A06	2580705	2581142	-	119	13449.43	44.54	6.73	-0.167	2,1	Plasma Membrane
BraA06g004330.3C	*B.rapaGH3-28*	A06	2587117	2590916	-	579	65024.03	35.28	5.43	-0.227	3,2	Plasma Membrane
BraA06g007430.3C	*B.rapaGH3-29*	A06	4122197	4124219	-	559	62678.08	43.21	5.57	-0.004	4,3	Plasma Membrane
BraA06g007440.3C	*B.rapaGH3-30*	A06	4125651	4127665	-	573	64176.46	42.81	5.44	-0.062	4,3	Plasma Membrane
BraA06g007450.3C	*B.rapaGH3-31*	A06	4132550	4133068	-	172	19215.33	43.17	7.59	0.147	1,0	Plasma Membrane
BraA06g007460.3C	*B.rapaGH3-32*	A06	4133210	4134532	-	380	42296.39	38.98	4.98	0.031	3,2	Plasma Membrane
BraA07g002570.3C	*B.rapaGH3-33*	A07	1940476	1943326	-	598	67670.59	46.45	5.23	-0.096	5,4	Plasma Membrane
BraA07g011840.3C	*B.rapaGH3-34*	A07	11542028	11544640	-	608	68907.2	45.95	5.05	-0.314	4,3	nuclear
BraA08g011040.3C	*B.rapaGH3-35*	A08	9616921	9617361	-	146	16446.83	39.34	6.81	-0.281	1,0	Extracellular
BraA08g011050.3C	*B.rapaGH3-36*	A08	9617371	9625110	-	452	50850.76	48.96	4.86	-0.208	4,3	Nuclear
BraA08g011080.3C	*B.rapaGH3-37*	A08	9687511	9691751	-	585	66475.29	35.93	5.7	-0.192	4,3	Nuclear
BraA08g026630.3C	*B.rapaGH3-38*	A08	18946334	18948847	-	585	66475.29	35.93	5.7	-0.192	4,3	Plasma Membrane
BraA09g002530.3C	*B.rapaGH3-39*	A09	1600527	1603208	-	592	66679.97	37.63	6.07	-0.245	4,3	Plasma Membrane
BraA09g010220.3C	*B.rapaGH3-40*	A09	5885146	5887133	-	590	66885.45	53.2	5.78	-0.273	2,1	Nuclear
BraA09g018240.3C	*B.rapaGH3-41*	A09	11548459	11550738	+	558	62762.99	45.01	5.82	-0.112	4,3	Plasma Membrane
BraA09g036560.3C	*B.rapaGH3-42*	A09	28947229	28950147	-	609	69105.51	46.65	5.08	-0.306	4,4	Nuclear
BraA09g054360.3C	*B.rapaGH3-43*	A09	39325121	39327378	+	615	70147.85	48.8	6.6	-0.25	3,2	Plasma Membrane
BraA09g061810.3C	*B.rapaGH3-44*	A09	43043729	43045811	+	572	64127.12	44.71	5.29	-0.128	4,3	Plasma Membrane

BraA09g061820.3C	*B.rapaGH3-45*	A09	43049696	43053735	+	572	63967.03	38.94	5.31	-0.118	4,3	Plasma Membrane
BraA09g061830.3C	*B.rapaGH3-46*	A09	43055184	43057173	+	570	63713.09	37.28	5.2	-0.039	4,3	Plasma Membrane
BraA10g012800.3C	*B.rapaGH3-47*	A10	10533010	10535110	-	561	63002.2	44.8	5.6	-0.071	4,3	Plasma Membrane
BraA10g025420.3C	*B.rapaGH3-48*	A10	17104827	17107294	-	594	67082.34	44.74	7.81	-0.135	4,3	Plasma Membrane
BraA10g025440.3C	*B.rapaGH3-49*	A10	17113087	17119301	-	750	84824.15	44.96	4.99	-0.477	6,5	Nuclear

Table 2. Ka/Ks values for duplicated genes in *B. rapaGH3*.

Gene 1/ Gene 2	Ka	Ks	Ka_Ks	T(MYA)
BraA01g018010.3C/ BraA03g053600.3C	0.01641	0.342937	0.047853	11.43122198
BraA07g011840.3C/ BraA09g036560.3C	0.034344	0.272981	0.12581	9.099374162
BraA08g011080.3C/ BraA08g011050.3C	0.00385	0.009605	0.400808	0.320175134
BraA03g005710.3C/ BraA07g002570.3C	0.098438	0.399046	0.246683	13.30154858
BraA09g002530.3C/ BraA03g043000.3C	0.076756	0.394271	0.194678	13.14238174
BraA04g032040.3C/ BraA05g001430.3C	0.051543	0.471933	0.109216	15.73108446
BraA06g004330.3C/ BraA06g004320.3C	0.118793	0.269646	0.440551	8.988200323
BraA03g005690.3C/ BraA10g025440.3C	0.056854	0.423157	0.134356	14.10524836
BraA03g057960.3C/ BraA03g057970.3C	0.048929	0.118506	0.412881	3.950183866
BraA02g015820.3C/ BraA03g015460.3C	0.060621	0.349775	0.173314	11.6591639
BraA10g012800.3C/ BraA09g018240.3C	0.082925	0.237222	0.349565	7.907416134
BraA03g020350.3C/ BraA04g020010.3C	0.103898	0.249387	0.416614	8.312885885
BraA06g007430.3C/ BraA06g007460.3C	0.086831	0.241437	0.35964	8.047907764
BraA09g061810.3C/ BraA09g061820.3C	0.073008	0.140858	0.518311	4.695274339
BraA08g011040.3C/ BraA01g021520.3C	0.062667	0.234449	0.267295	7.814960593

3.4. Synteny analysis of the *B. rapaGH3* gene family among closely related species

Chromosomal synteny provides key evidence for reconstructing genome evolution by comparing conserved gene order among related species (Tang et al. 2008; Lyons et al. 2008). In Brassicaceae, such analyses are particularly valuable for refining gene annotations and tracing consequences of whole genome duplications and triplications (Cheng et al. 2012). In this study, synteny of the *B. rapaGH3* gene family was examined by comparing the *B. rapa* genome with the reference genome of *Arabidopsis thaliana* and with other *Brassica* species.

Collinearity analysis revealed conserved *GH3* orthologs linking the 49 *B. rapaGH3* genes to 26 genes in *A. thaliana*, 29 in *B. nigra*, 31 in *B. oleracea*, 41 in *B. napus*, 39 in *B. juncea*, and 32 in *B. carinata* (Fig. 5). Species were selected based on their morphological and genomic similarity to *B. rapa* and their shared ancestry with *Arabidopsis*, as described in U's triangle (Cheng et al., 2014). The strongest collinearity was observed between *B. rapaGH3* genes and those of *B. napus*, followed by *B. juncea*, *B. carinata*, *B. oleracea*, *B. nigra*, and *A. thaliana*. Notably, *B. rapa* A genome *GH3* homologs were retained in the A, B, and C sub genomes of all polyploid Brassica species examined, indicating extensive conservation of *GH3* loci after whole genome triplication. These results support the view that recurrent genome-wide and segmental duplication events have expanded and redistributed *GH3* genes within Brassicaceae, while maintaining their core syntenic relationships across species.

3.5. *Cis*-element Analysis in Promoter Regions of *B. rapaGH3* Genes and Their Distribution

The *cis*-regulatory elements present in the promoter regions of *B. rapaGH3* genes were analyzed to gain insight into their potential roles in plant growth, development, and responses to phytohormones. The complete dataset of identified *cis*-elements are provided in Table S1. These elements were classified into three major categories: growth and development-related elements, phytohormone-responsive elements, and abiotic stress-responsive elements. In total, six *cis*-elements associated with abiotic stresses, including drought, light, low temperature, wounding, defense and stress, and anoxic conditions were identified (Fig. 6).

In addition, five growth- and development-related elements were detected, which are involved in the regulation of the cell cycle, meristem expression, endosperm expression, circadian control, and zein metabolism. These findings indicate that *B. rapaGH3* genes may play complex roles across different developmental stages. Notably, some key *cis*-elements were found to be gene-specific, whereas others were widely and randomly distributed among multiple *GH3* genes. Importantly, the expression of *B. rapaGH3* genes may be influenced by developmental stages, phytohormone signaling, and exposure to various abiotic stress conditions.

Overall, five phytohormone-responsive elements, abscisic acid (ABA), methyl jasmonate (MeJA), gibberellin (GA), salicylic acid (SA), and auxin, and four abiotic stress-responsive elements related to temperature, drought, light, and salinity were identified (Fig. S4). Most abiotic stress-related elements were randomly distributed across the promoters, with some restricted to a limited number of genes, highlighting their potential roles in stress tolerance. Gene-specific distribution patterns were observed for certain elements. Among all *B. rapaGH3* promoters, auxin- and light-responsive elements were the most abundant, whereas salicylic acid-responsive elements were the least frequent. These results suggest that genes harboring element-specific *cis*-regulatory patterns may serve as promising candidates for further functional studies aimed at elucidating their roles in hormone-mediated stress responses.
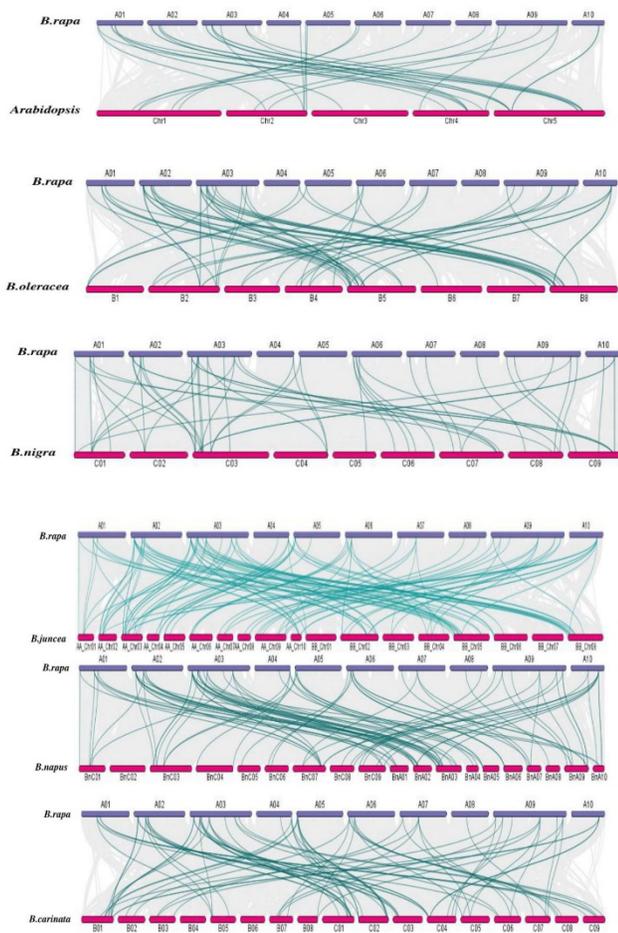
Fig 5. Syntenic relationship between *Brassica* species and *Arabidopsis*.

3.6. Prediction of the 3D Structures of *B. rapaGH3*s

Three-dimensional (3D) structural modeling was performed to investigate the structural characteristics of the *B. rapaGH3* protein family. A total of 49 *B. rapaGH3* proteins were analyzed, and all were predicted to share a conserved 3D architecture primarily composed of α-helices and β-sheets (Fig 7). The high degree of structural similarity among *B. rapaGH3* proteins indicates strong structural connectivity within the family and suggests functional conservation. As proteins with similar tertiary structures often exhibit related biological functions, the conserved folding patterns observed here imply that all *B. rapaGH3* members retain the characteristic monomeric structure of the *GH3* family. The N- to C-terminal regions of the modeled proteins are represented using a rainbow color scheme to illustrate structural continuity. All protein models were generated with 100% confidence using the c2khoA template. Domain analysis confirmed the presence of the conserved *GH3* domain (PF03321.16) in all *B. rapaGH3* proteins. Consistent with previous studies, these proteins exhibit the hallmark *GH3* family features, including a mixed β-sheet core flanked by α-helices, resulting in a stable monomeric fold with a characteristic (αβα) topology (Abbas et al., 2022). Overall, the predicted tertiary structures of *B. rapaGH3* proteins demonstrate conserved structural organization, support functional relatedness despite sequence divergence, and highlight their shared biological roles.

3.7. Genome-wide Analysis of miRNA-associated *B. rapaGH3* Genes

To further elucidate the regulatory mechanisms of *B. rapaGH3* genes, their potential interactions with microRNAs (miRNAs) were investigated. A total of 34 distinct miRNA subtypes were identified as putative regulators of *B. rapaGH3* genes. Among them, 12 miRNAs were predicted to target *B.*

rapaGH3-38, while the number of miRNAs targeting other *GH3* genes varied considerably (Fig 8A; Table S2).

Previous studies have shown that miRNAs regulate gene expression by binding to complementary regions of target mRNAs (Jamla et al., 2022), a mechanism consistent with the predicted interactions observed in this study (Fig. 8B). Detailed information on miRNA-target binding sites is provided in Table S2, which also indicates that multiple miRNAs can target the same *B. rapaGH3* gene.

Although these interactions were predicted in silico, further experimental validation is required to confirm the expression patterns of the identified miRNAs and their corresponding target genes in *B. rapa*, as well as to clarify their biological roles.
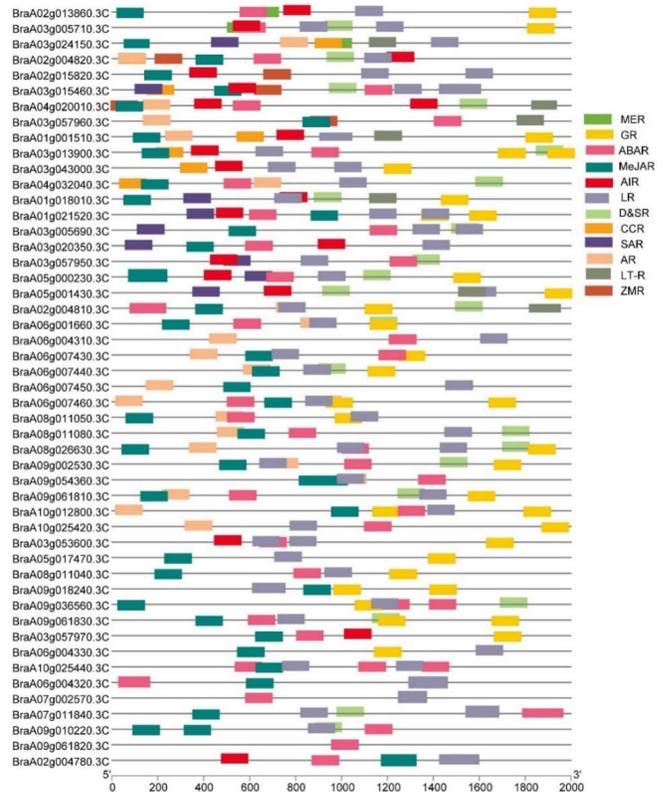

Fig 6. Identified *cis*-acting elements in *B. rapaGH3* genes.

3.8. KEGG analysis and Transcriptomic Analysis of *B. rapaGH3* Genes Along with Their Orthologues in *Arabidopsis*

KEGG pathway enrichment study discovered three pathways participating in diverse functions of *B. rapaGH3* genes. The highly enriched pathways include Plant hormone signal transduction, Signal transduction, auxin-responsive *GH3* gene family, and environmental information processing. In summary, the KEGG enrichment study reinforces the functional significance of *B. rapaGH3* in attenuating defense responses and developmental pathways that are associated with signal transduction and environmental changes.

B. rapa 'Chiifu-401-42' variety RNA-seq data (NCBI Gene Expression Omnibus; accession no. GSE43245) were used to investigate the transcriptome expression profile of *B. rapaGH3* genes. Transcriptomic analysis revealed that 35 out of 49 B. rapaGH3 genes were expressed in 35-day-old seedlings. Fig 9A demonstrated that *B. rapaGH3-3, B. rapaGH3-21, B. rapaGH3-23, B.rapaGH3-25, B.rapaGH3-31, B.rapaGH3-32, and B.rapaGH3-35 expressing higher as compared to remaining. B. rapaGH3-4, B. rapaGH3-6, B. rapaGH3-16, B.rapaGH3-18, B.rapaGH3-34, B.rapaGH3-41, B.rapaGH3-25, B.rapaGH3-46, B.rapaGH3-47, and B.rapaGH3-48* showed no change in expression (Fig 9A).
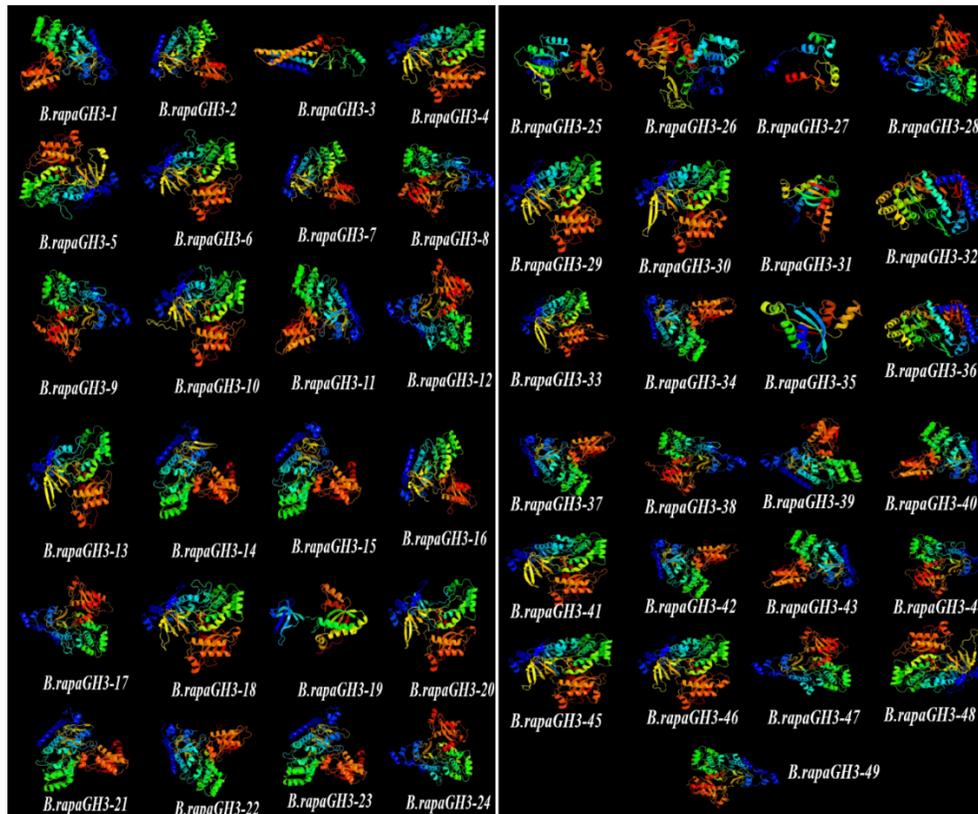
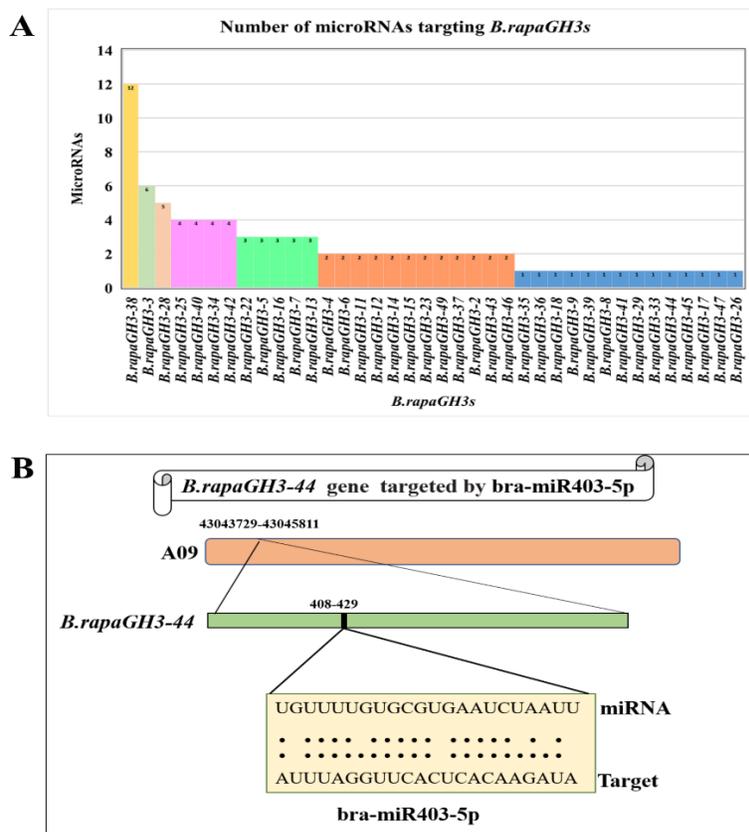**Fig 7**. Predicted three-dimensional structure of *B. rapaGH3*

Fig 8. miRNA targeting *B.rapaGH3* genes. (A) Graphical illustration of the number of miRNAs targeting their respective *B.rapaGH3* genes. (B) The graphic illustration indicates *B.rapaGH3*-44 gene is targeted by bra-miR403-5p. The gene's chromosomal location is indicated by an orange thick bar. The position of miRNAs on the gene sequence is indicated by the green thick bar, and the complementary site of the RNA sequence is depicted in a yellow box.

4. Discussion

The accessibility of open-source whole-genome sequence databases has enabled comprehensive analyses of *GH3* gene families across a wide range of plant species, including *Arabidopsis*, rice, wheat, and maize. Furthermore, these studies have demonstrated the important roles of *GH3* genes in plant growth, development, and defense mechanisms (Fu et al., 2011, Khan and Stone 2007b, Kong et al. 2019, Zhang et al. 2016). In this study, a genome-wide analysis was conducted in *B. rapa*. The genome-level findings provide valuable insights and serve as a reference framework for future functional investigations (Safder et al., 2022). The identification of 49 *GH3* genes in *B. rapa* suggests that this family has expanded through gene duplication, a process that plays an essential role in the diversification and functional specialization of gene families in plants (Magadum et al., 2013). Gene duplication events, particularly segmental and tandem duplications, have likely played a key role in shaping the evolutionary trajectory of *GH3* genes in *B. rapa*. This conclusion is further supported by Ka/Ks ratio analyses, which indicate that these genes are predominantly under purifying selection, reflecting strong evolutionary conservation and functional constraint (Guéguen and Duret, 2018). Such evolutionary evidence suggests that *GH3* genes maintain essential roles in auxin homeostasis, consistent with previous findings in *Arabidopsis* and *Brassica oleracea* (Cheng et al., 2014).

4.1. *B.rapaGH3* Gene Family Characterization

Arabidopsis thaliana and *Brassica rapa* both belong to the family Brassicaceae. In this study, we identified 49 members of the *GH3* gene family in *B. rapa*. The physical characteristics of these genes were examined, revealing that while exon–intron organization was generally conserved, notable variation existed in intron length and overall gene sequence among the identified *GH3* genes. The conservation of exon–intron structures and motif composition within individual phylogenetic subgroups suggests functional conservation among *B. rapaGH3* genes. Genes clustered within the same evolutionary group exhibited similar exon–intron architectures and conserved motif patterns (Fig. S1 and Table 1), supporting the reliability of the phylogenetic classification. Such structural conservation is often associated with shared biological functions. Previous studies have demonstrated that genes with fewer introns tend to be expressed more rapidly and at higher levels in plants (Jeffares et al., 2008). Consistent with this observation, *B. rapaGH3* genes containing fewer introns may exhibit enhanced expression, particularly under stress conditions where rapid transcriptional responses are advantageous. This hypothesis warrants further experimental validation, such as quantitative real-time PCR (qRT-PCR) analysis under diverse abiotic stress treatments. To further explore the evolutionary relationships among *GH3* genes, a phylogenetic tree was constructed including *B. rapa*, other Brassica species, and A. thaliana. The presence of *GH3* orthologs across these species supports a shared evolutionary origin and highlights the conserved nature of the *GH3* gene family within the Brassicaceae lineage (Fig. 3). The evolutionary relationships inferred from phylogenetic analysis indicate that *B. rapaGH3* genes are highly conserved with their homologs in other Brassicaceae species, reflecting a shared evolutionary origin. This observation is consistent with the well-established U's Triangle model, which describes the genomic relationships and evolutionary history among Brassica species (Cheng et al., 2014).

Furthermore, the presence of *GH3* orthologs across multiple species suggests a degree of functional redundancy and underscores the essential roles of these genes in fundamental plant developmental processes and stress responses. To further elucidate their precise biological functions, additional functional studies such as gene knockout or overexpression analyses will be necessary to validate the roles of *GH3* genes across different plant species. Factors that are mainly responsible for the evolution of plant genomes in length and structure tend to be gene duplications and chromosomal segments (Liu et al., 2010). Segmental or tandem duplication events refer to genetic duplication events, and these occur when two or more neighboring gene copies are attached, either on the same chromosome or on different chromosomes (Cannon et al., 2004). Gene duplication events were

investigated due to their importance in understanding the evolutionary expansion of the GH3 gene family. Gene duplication could provide new genetic information on which mutations, displacements, and screening can develop, resulting in highly specialized or new genetic alterations (Long et al., 2003). The variation of a genome or species in adjusting to shifting surroundings would be greatly constrained in the absence of gene duplication (Magadum et al., 2013). The function of a duplicate, its mode of duplication (whether it was distributed during a whole-genome duplication event), the species in which it happens, and its expression rate influence whether it is retained. Exaptation of maintaining existing specific functions is an essential characteristic of gene evolution and morphological evolution (Magadum et al., 2013). Syntenic fragments shared by different species enable the deduction of the evolutionary processes of convergent evolution and may even provide insights into the mechanisms underlying these processes (Feng et al., 2015). The role of gene duplication in the evolution of the *B. rapaGH3* gene family is further supported by synteny analysis comparing conserved syntenic blocks between *B. rapa* and related species. These results indicate that duplication events within the *GH3* family have been maintained throughout evolutionary history, underscoring their essential roles in auxin regulation and plant stress responses (Tang et al., 2008). Moreover, the widespread occurrence of both tandem and segmental duplications among *GH3* genes suggests that these genes have been subjected to selective pressure, likely to fine-tune protein functions in response to diverse environmental stimuli. Such duplication-driven diversification may have contributed to the functional specialization of *GH3* proteins while preserving their core biological roles.
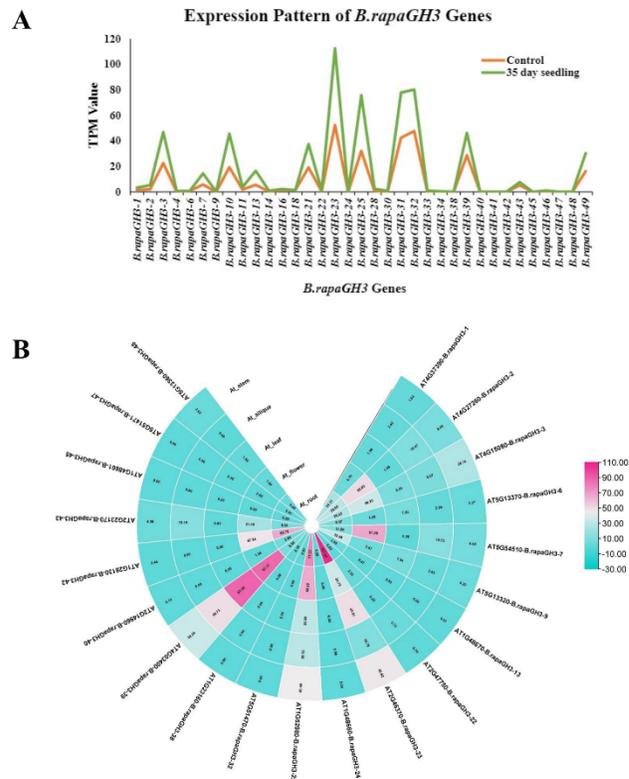
Fig 9. A Expression pattern of *B. rapaGH3* genes in Transcripts Per Kilobase Million (TPM) in 35 day seedlings of *B.rapa*. (B) Expression pattern of *Arabidopsis* homologues *GH3* genes in different tissues.

4.2. miRNA: Key Players in Gene Function

microRNAs such as bra-miR408-3p may play key roles in post-transcriptional regulation of *B.rapaGH3* genes, modulating their expression during stress responses (Statello et al., 2021). Numerous miRNAs that respond to various developmental conditions have been identified in *B. rapa* in recent years (Wang et al. 2019, Yu et al. 2012). These results indicate that Brassica-associated miRNAs might well play essential roles in stress resistance by

adapting the transcript levels of *GH3* genes. In our study, we found that bra-miR9554-3p, bra-miR161-3p, bra-miR408-3p, bra-miR6032-3p, bra-miR5713, bra-miR400-5p, bra-miR172a, bra-miR159a, bra-miR5717, bra-miR395a-5p, bra-miR168a-5p, c-5p, and bra-miR5720 (Table. S2). Previous research has also proven the significance of these microRNAs in the proper function of their target genes (Wang et al., 2012; Jiang et al., 2013). Future studies should consider functional validation of *B.rapaGH3* genes using CRISPR/Cas9 or RNA interference to explore their roles in auxin signaling and stress responses.

These regulatory molecules indicated here help fine-tune the expression of *GH3* genes in *B. rapa* during developmental processes and stress responses, whereby 34 miRNAs were potentially targeting the *GH3* genes. Indeed, control of *B. rapaGH3*-38 is regulated by the highest number of miRNAs, reaffirming its significance in monitoring the auxin-related processes. These miRNA-gene relations can be tested for validation in an experimental manner by methods like miRNA inhibition or overexpression assays. It would also bring out more understanding of how these miRNAs are involved in the regulation of plant responses under different conditions.

4.3. Functional Determination and Digital Expression Analysis of *B.rapaGH3* Genes

Promoter analysis revealed that *GH3* genes harbor *cis*-acting elements responsive to phytohormones, abiotic stress, defense, and light, indicating their potential involvement in regulating plant responses to fluctuations in light intensity and temperature. KEGG pathway analysis further demonstrated the association of *GH3* genes with signal transduction and hormone-mediated regulatory pathways. KEGG enrichment highlighted the role of *GH3* genes in auxin signaling, suggesting that they may modulate plant responses to abiotic stresses such as drought and salinity through hormone crosstalk (Zhao, 2010).

To clarify the underlying regulatory mechanisms, future studies should focus on detailed molecular and functional characterization of *GH3* genes in Brassica species. The identification of light- and salicylic acid-responsive *cis*-elements in *B. rapaGH3* promoters suggests potential interactions with transcription factors such as TGA and WRKY, which are known to regulate plant defense and developmental processes under environmental stress conditions (Liu et al., 2010). Several studies have employed qRT-PCR and transcriptomic approaches to examine expression patterns of *GH3* family genes; however, only a limited number of *GH3* genes have been functionally characterized across plant species. Based on comprehensive in silico analyses and digital transcriptomic data from *B. rapa* and other plants, we present a preliminary framework for elucidating the structural and functional characteristics of the *B. rapaGH3* gene family. These findings provide valuable resources for crop improvement strategies and may be exploited in breeding programs aimed at enhancing stress resistance in Brassica crops, particularly through gene-editing technologies such as CRISPR/Cas9 or marker-assisted selection.

Promoter analysis further revealed a high abundance of light-responsive *cis*-acting elements, indicating a potential role for *GH3* genes in photomorphogenesis and plant developmental regulation. This observation is consistent with previous studies in *Arabidopsis*, where light and phytochrome signaling pathways have been shown to regulate *GH3* gene expression (Park et al., 2007). Moreover, the presence of hormone- and stress-responsive *cis*-elements highlights the multifaceted roles of *GH3* genes in integrating endogenous hormonal cues with environmental signals. Future functional validation using approaches such as promoter-reporter assays would help confirm the regulatory activity of these *cis*-elements under varying light and stress conditions.

5. Conclusion

In this study, a genome-wide analysis of the *GH3* gene family in *B. rapa* identified 49 putative *GH3* genes for further investigation. Using a range of in silico approaches, we explored the evolutionary characteristics of the *B. rapaGH3* gene family, including gene structure, chromosomal distribution, duplication events, and synteny relationships. In addition, analyses of targeted microRNAs, promoter *cis*-acting regulatory elements, and KEGG pathways were performed. The results indicate that *B. rapaGH3* genes play important roles in plant defense mechanisms, exhibit high sensitivity to light, temperature, and phytohormone signaling, and are regulated by multiple miRNA families. Furthermore, predicted three-dimensional protein structures provide insights into their functional properties, which may contribute to crop improvement, enhanced stress tolerance, and increased biomass yield. Transcriptome-based digital expression analyses further demonstrated that *GH3* genes are strongly involved in the growth and developmental processes of *B. rapa*. Overall, this comprehensive analysis of the *GH3* gene family establishes a solid foundation for future studies aimed at elucidating the molecular functions and physiological roles of these genes in Brassica crops.

Author Information

Corresponding Author

Javaria Tabusam: State Key Laboratory of Cotton Bio- breeding and Integrated Utilization, Institute of Cotton Research, Chinese Academy of Agricultural Science, Anyang, China.
Email: javariatabusam77@gmail.com
ORCID: https://orcid.org/0000-0003-0760-6139

Author(s)

Yixing Zhang: College of Agronomy, Hebei Agricultural University, Baoding 071001, China.
ORCID: https://orcid.org/0009-0000-4827-9539

Yi Ren: National Engineering Research Center for Agriculture in Northern Mountainous Areas, Hebei Agricultural University, Baoding, Hebei 071001, China.
ORCID: https://orcid.org/0000-0002-3076-0268

Author Contributions

JT: conceptualization, methodology, validation, supervision, formal analysis, investigation, software, writing original draft, writing, review and editing; JT, YZ, and YR: conceptualization, methodology, visualization, writing review and editing, investigation, data curation, software, formal analysis; All authors contributed to the research article and approved the final version.

Competing Interests

The authors have no relevant financial or non–financial interests to disclose.

Data availability

All data generated or analyzed during this study are included in this article and the Supplementary Material.

Additional Information

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