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ARTICLE

Genome-Wide Identification, Evolution and Expression Analyses of *GA2ox* Gene Family in *Brassica napus* L.

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ABSTRACT

Gibberellin 2-oxidases (*GA2ox*) are important enzymes that maintain the balance of bioactive GAs in plants. *GA2ox* genes have been identified and characterized in many plants, but these genes were not investigated in *Brassica napus*. Here, we identified 31 *GA2ox* genes in *B. napus* and 15 of these *BnaGA2ox* genes were distributed in the A and C subgenomes. Subcellular localization predictions suggested that all *BnaGA2ox* proteins were localized in the cytoplasm, and gene structure analysis showed that the *BnaGA2ox* genes contained 2–4 exons. Phylogenetic analysis indicated that *BnGA2ox* family proteins in monocotyledons and dicotyledons can be divided into four groups, including two C_{19}-GA2ox and two C_{20}-GA2ox clades. Group 4 is a C_{20}-GA2ox Class discovered recently. Most *BnaGA2ox* genes had a syntenic relationship with *AtGA2ox* genes. *BnaGA2ox* genes in the C subgenome had experienced stronger selection pressure than genes in the A subgenome. *BnaGA2ox* genes were highly expressed in specific tissues such as those involved in growth and development, and most of them were mainly involved in abiotic responses, regulation of phytohormones and growth and development. Our study provided a valuable evolutionary analysis of *GA2ox* genes in monocotyledons and dicotyledons, as well as an insight into the biological functions of *GA2ox* family genes in *B. napus*.

KEYWORDS

Brassica napus; *GA2ox* gene family; evolution; expression patterns

1 Introduction

Gibberellins (GAs) are diterpenoid compounds with biological activity in plants. They are factors that help to regulate plant growth and development, including seed germination, fruit development, flowering time, leaf morphology and internode elongation [1,2]. The biosynthetic pathways and the main regulatory mechanisms of GAs are understood in *Arabidopsis thaliana* [3–5]. Most of the genes involved in GAs biosynthesis and regulation of GAs have been cloned in plants such as *A. thaliana* [6], rice (*Oryza sativa*) [7] and pea (*Pisum sativum*) [8]. Deletion or mutation of the genes associated with the biosynthesis of GAs can result in dwarf or semi-dwarf plant phenotypes [8–11]. GAs have been manipulated in crop

plants to increase elongation of stems in sugarcane (*Saccharum officinarum*) and retard seed growth in cotton (*Gossypium hirsutum*) [11].

GA2ox, GA3ox and GA20ox are key oxidases in GA biosynthesis. They belong to the 20G-Fe (II) oxygenase superfamily, and their deletion or mutation can cause dwarf or semi-dwarf plant phenotypes [8–12]. According to the number of carbon (C) atoms, GAs are classified into different groups, including C_{19}-GAs (such as GA_9, GA_{20}, GA_1, and GA_4), and C_{20}-GAs (such as GA_{12}, GA_{15}, GA_{24}, GA_{53}, GA_{44}, and GA_{19}). GA2-oxidases can act on bioactive GA_1 and GA_4 to affect C-2 hydroxylation to transform them into inactive GA_8 and GA_{34}, and the GA2ox proteins are also classified into C_{19}-GA2ox or C_{20}-GA2ox according to its roles for C_{20}-GA or C_{19}-GA substrates. However, the synthesis of active GA_1 and GA_4 depends on GA_{12} and GA_{53} [2,11,13]. GA2ox is a key factor maintaining the balance between bioactive GAs and intermediates in plants [14]. It was first identified in bean (*Phaseolus coccineus*) [15] and then identified in other plants such as rice [16,17], *A. thaliana* [11,18], maize (*Zea mays*) [19], grape (*Vitis vinifera*) [20] and tea tree (*Camellia lipoensis*) [21]. Overexpression of *GA2ox* genes without tissue or species-specific regulation results in growth and reproductive abnormalities such as dwarfism and sterility. For example, overexpression of bean *GA2ox1* in rye (*Secale cereale*) and ryegrass (*Lolium perenne*) resulted in a dwarf phenotypes owing to the reduction of bioactive GA_1 and GA_4 in the transgenic plants [15]. Similar dwarf phenotypes have been observed in tobacco (*Nicotiana tabacum*) by overexpressing tea *GA2ox1* or *GA2ox3*. These transgenic plants showed delayed flowering, reduced growth, and smaller, rounder and darker green leaves [21]. In addition to the regulation of plant height, *GA2ox* genes also affect responses to abiotic stresses and to photomorphogenesis. Overexpression of *OsGA2ox5* improved resistance to high salt in rice [22]. Induction of *GA2ox* expression reduced bioactive GA_4 and promoted photomorphogenesis in *A. thaliana* [23]. Some AtGA2ox proteins also function in catalyzing the binding of certain phytohormones to amino acids, such as indoleacetic acid and salicylic acid [24,25].

B. napus (rapeseed) is an important oilseed crop worldwide and is formed from two diploid species *Brassica rapa* and *B. oleracea* [26,27]. Ectopic expression of *B. napus GA2ox6* in *A. thaliana* resulted in late flowering and increased chlorophyll content in leaf [28]. This suggested that the GA pathway regulation via the *GA2ox* genes may have potential for *B. napus* improvement. However, there are little studies focused on the biosynthetic activity of *GA2ox* genes in *B. napus* compared to rice and wheat, and the *GA2ox* family genes in *B. napus* still remain unknown. Here, we identified *BnaGA2ox* genes using a reciprocal Basic Local Alignment Search Tool Protein (BLASTP) with *AtGA2ox* genes as query sequences. We analyzed their protein characters and gene structure, as well as the cis-elements in promoter regions. We also examined the evolution among *GA2ox* members from seven species and investigated the spatiotemporal expression patterns of *BnaGA2ox* genes. The results provide insights into the evolution of GA2ox proteins in plants and help to elucidate the roles of *GA2ox* in *B. napus*.

2 Materials and Methods

2.1 Data Acquisition and GA2ox Genes Identification

The sequence files, including genomic, protein, coding sequences of *A. thaliana* were obtained from the Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), and that information from *B. oleracea* (JZS) and *B. rapa* (Chiifu) was obtained from the Brassicaceae Database (http://brassicadb.cn). The *B. napus* pan-genome Information Resource (BnPIR, http://cbi.hzau.edu.cn/bnapus/) was used to retrieve the sequences files of *B. napus* (ZS11), and the sequence files of tomato (*Solanum lycopersicum*) were downloaded from the Solanaceae Database (https://solgenomics.net). Sequence files of *O. sativa* (Japonica) and *Z. mays* (Zm-B73) were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/t).

GA2ox genes were identified following two steps: First, using nine GA2ox protein sequences from *A. thaliana* as query sequences, the candidate *GA2ox* genes were identified in a local reciprocal BLASTP

[29] with the screening parameters e-value < 1e-5 and qcover ≥50% [30]. Then, all candidate GA2ox proteins were studied through the online PfamScan (http://www.ebi.ac.uk/Tools/pfa/pfamscan/) to validate their functional domain of 2OG-FeII_Oxy (PF03171.20).

2.2 Protein Sequence Analysis

The online website ExPASy (http://web.expasy.org/protparam/) was used to calculate and predict the characteristics of BnaGA2ox proteins, including molecular weight (MW, kDa), number of amino acids (AA) and isoelectric point (pI). The SignalP 5.0 server was (http://www.cbs.dtu.dk/services/SignalP/) used to predict each BnaGA2ox protein signal peptide with default parameters, and their subcellular localizations were analyzed through the Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) with default parameters.

2.3 Phylogenetic and Evolution Analysis

The multiple GA2ox protein sequences were aligned by MUSCLE software [31] with default arguments. The Molecular Evolutionary Genetics Analysis (MEGA) 7 program [32] was used to construct the phylogenetic trees using the Neighbor-Joining (NJ) method, with the patterns setting to number of different + G+I substitution model + 1,000 bootstrap replications. The phylogenetic tree was displayed and modified using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). To compare the selective pressure on *GA2ox* genes, the coding sequence alignments between *B. napus* and *A. thaliana* were imported into KaKs_Calculator 2.0 [33] to evaluate the non-synonymous mutation rate (Ka) and synonymous mutation rate (Ks) with GY-HKY model.

2.4 Gene Structure and Protein Motif Identification Analysis

The exon/intron structures of *BnaGA2ox* were displayed using the Gene Structure Display Server (GSDS2.0, http://gsds.gao-lab.org/). The conserved motifs of BnaGA2ox proteins were identified using the online Multiple Em for Motif Elicitation (MEME, https://meme-suite.org/meme/tools/meme) through the following arguments: (1) optimum width of motif ranging from 6 to 100; (2) maximum number of motif sets to 10; and (3) e-value > 1e-10. The conserved motifs were displayed using TBtools [34].

2.5 Chromosomal Location, Syntenic Relationships and Cis-Acting Elements

The chromosomal locations of *BnaGA2ox* were obtained based on the annotation file in *B. napus* of ZS11 from BnPIR. The syntenic relationships of *GA2ox* between *B. napus* and *A. thaliana* were acquired from the Brassicaceae database (http://brassicadb.org/brad/), and displayed using TBtools. To analyze the *cis*-acting elements in *BnaGA2ox* promoter regions, the 1500-bp genomic DNA sequences upstream of *BnaGA2ox* genes were obtained using TBtools and submitted to the online PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.6 Expression Patterns of BnaGA2ox Genes and Quantitative Reverse-Transcription PCR

To compare and analyze the expression levels of *BnaGA2ox* genes in multiple tissues, we downloaded their expression files in cultivar ZS11 from *B. napus* transcriptome information resource (BnTIR, http://yanglab.hzau.edu.cn/BnTIR) [35]. There were 91 different tissues used in this study including roots, stems, leaves and sepals, petals, pollen, siliques and seeds at different stages (a detailed schematic diagram of tissues is shown in Supplementary Fig. S1). Meanwhile, we also downloaded the expression patterns of *BnaGA2ox* genes under phytohormone and abiotic treatments at the seedling leaves (SLs) and seedling roots (SRs) from BnTIR, including seven phytohormones and six abiotic treatments. All gene expression levels of *BnaGA2ox* genes were normalized by Log_2(TPM+1).

To verify the RNA-seq results, four genes (*BnaGA2ox1a*, *BnaGA2ox2a*, *BnaGA2ox6d*, *BnaGA2ox9a*) were selected for qRT-PCR analysis. Total RNA was extracted from tissues using an RNA Extraction Kit (Tiangen, Beijing, China), including roots, stems, leaves and buds at the bolting stages, and seeds at different stages. Their cDNA was synthesized according to the manufacturer's instructions using M-MLV transcriptase (TaKaRa Biotechnology, Dalian, China). A Bio-Rad CFX96 Real Time System (USA) was used to perform the Reverse transcription quantitative PCR (RT-qPCR), and gene-specific primers were designed using Geneious 10 (Biomatters, Auckland, New Zealand) (Supplementary Table S1). The qRT-PCR reactions were performed as described in the MIQE guidelines [36], with three technical replicates for each sample. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with *BnACT7* as internal controls.

3 Results

3.1 Identification and Characterization of BnGA2ox Genes

We found 10 *GA2ox* genes in *A. thaliana* by searching the GA2ox name from TAIR. However, *AtGA2ox5* (*AT3G17203*) is a pseudogene. Using the remaining nine AtGA2ox protein sequences as query, a total of 31 *BnaGA2ox* genes were identified using a combination between the reciprocal BLASTP and 2OG-FeII_Oxy domain screening (Table 1). We named the putative *BnaGA2ox* genes according to their homologous *AtA2ox* genes. For most of the *AtGA2ox* genes, two to five homologous *BnaGA2ox* genes were identified; however, no homolog was identified for *AtGA2ox10* in *B. napus* genome. Among all BnaGA2ox proteins, the number of amino acids ranged from 213 (BnaGA2ox7b) to 379 aa (BnaGA2ox9c), with an average of 327.6 aa. The Mw (molecular weights) were found to range from 24.63 (BnaGA2ox7b) to 42.47 kDa (BnaGA2ox9c), with an average of 36.99 kDa, and the pI (isoelectric point) ranged from 4.80 (BnaGA2ox9c) to 8.98 (BnaGA2ox7b), with an average of 6.84 (Table 1). All BnaGA2ox proteins were suggested to be localized in the cytoplasm, while no signal peptide was observed in any BnaGA2ox protein (Table 1). In addition, using the same methods, we also identified 12 *GA2ox* genes in *O. sativa*, 16 *GA2ox* genes in *Z. mays*, 11 *GA2ox* genes in *S. lycopersicum*, 17 *GA2ox* genes in *B. oleracea* and 17 *GA2ox* genes in *B. rapa* (Supplementary Table S2), respectively.

3.2 Phylogenetic Analysis of GA2ox Protein Members

To study the phylogenetic relationships of GA2ox proteins in *B. napus*, as well as in monocotyledons and dicotyledons, we constructed a NJ tree using GA2ox proteins from *B. rapa*, *B. oleracea*, *B. napus*, *A. thaliana*, *O. sativa*, *Z. mays* and *S. lycopersicum* (Fig. 1, Supplementary Table S2). The GA2ox proteins in these seven species were divided into four groups based on phylogenetic topology and bootstrap values (Fig. 1). They were named Group 1 (GA2ox1/2/3 subfamily), Group 2 (GA2ox4/6 subfamily), Group 3 (GA2ox7/8 subfamily) and Group 4 (GA2ox9/10 subfamily). Group 1 and Group 2 belong to the C_{20}-GA2ox Class, and Group 3 and Group 4 belong to the C_{19}-GA2ox Class. Among the four groups, the largest clade was Group 1, containing 47 GA2ox proteins, including 33 GA2ox proteins of Brassicaceae, and the smallest clade was Group 4, containing 13 GA2ox proteins, including 10 GA2ox proteins of Brassicaceae, and each clade presented at least one GA2ox member per plant. Further analysis revealed that the GA2ox members from *B. oleracea*, *B. rapa*, *B. napus*, *O. sativa* and *Z. mays* in Group 1 and Group 3 were larger than those in Group 2 and Group 4. This indicated an expansion of GA2ox members in the *Brassica* species of Group 1 and Group 3, as well as in the monocotyledons. In each group, the AtGA2oxs always first clustered with its orthologues from the *Brassica* to form a Brassicaceae cluster, and then clustered with the GA2oxs in *S. lycopersicum* to form a dicotyledon cluster that finally joined with GA2ox members in *O. sativa* and *Z. mays* to form a specific GA2ox clade (Fig. 1). These results suggest that the homology of GA2ox proteins in the species was consistent with previous perceived species relationships. We also hypothesized that there should be two ancient copies of GA2ox in the common ancestral species of monocotyledons and dicotyledons based on the GA2ox phylogenetic relationships. One as the ancestral copy for C_{20}-GA2ox Class and the other as the ancestral copy for C_{19}-GA2ox Class.

Table 1: The identification and characteristics of *GA2ox* genes in *B. napus*

Gene name	Gene ID	Orthologues in *A. thaliana*	Chromosome location	Number of amino acids	Molecular weights (Mw, kDa)	Isoelectric point (pI)	Subcellular localization
BnaGA2ox1a	*BnaA02G0232000ZS*	*AT1G78440 (AtGA2ox1)*	A02:15057125-15058865	320	35.62	6.54	Cytoplasm
BnaGA2ox1b	*BnaA07G0375200ZS*	*AT1G78440 (AtGA2ox1)*	A07:31554207-31555759	330	37.09	5.74	Cytoplasm
BnaGA2ox1c	*BnaA07G0375300ZS*	*AT1G78440 (AtGA2ox1)*	A07:31566808-31568534	324	36.219	5.88	Cytoplasm
BnaGA2ox1d	*BnaC02G0312300ZS*	*AT1G78440 (AtGA2ox1)*	C02:30578728-30580391	320	35.62	6.01	Cytoplasm
BnaGA2ox1e	*BnaC06G0441000ZS*	*AT1G78440 (AtGA2ox1)*	C06:51449609-51451269	328	36.64	5.36	Cytoplasm
BnaGA2ox2a	*BnaA07G0086100ZS*	*AT1G30040 (AtGA2ox2)*	A07:13696389-13698457	341	38.10	7.12	Cytoplasm
BnaGA2ox2b	*BnaA08G0206600ZS*	*AT1G30040 (AtGA2ox2)*	A08:22954785-22956650	333	37.53	8.44	Cytoplasm
BnaGA2ox2c	*BnaA09G0413400ZS*	*AT1G30040 (AtGA2ox2)*	A09:47444619-47446483	341	38.30	5.46	Cytoplasm
BnaGA2ox2d	*BnaC03G0661800ZS*	*AT1G30040 (AtGA2ox2)*	C03:63966076-63968008	335	37.73	8.44	Cytoplasm
BnaGA2ox2e	*BnaC07G0133200ZS*	*AT1G30040 (AtGA2ox2)*	C07:25541902-25544098	341	38.10	7.12	Cytoplasm
BnaGA2ox3a	*BnaA03G0165900ZS*	*AT2G34555 (AtGA2ox3)*	A03:8475272-8476994	339	38.38	8.37	Cytoplasm
BnaGA2ox3b	*BnaA05G0099800ZS*	*AT2G34555 (AtGA2ox3)*	A05:5842073-5843803	335	38.08	5.68	Cytoplasm
BnaGA2ox3c	*BnaC03G0192100ZS*	*AT2G34555 (AtGA2ox3)*	C03:11005243-11007342	336	38.20	8.36	Cytoplasm
BnaGA2ox3d	*BnaC04G0123900ZS*	*AT2G34555 (AtGA2ox3)*	C04:11222715-11225186	335	37.99	6.77	Cytoplasm
BnaGA2ox3e	*BnaC04G0534400ZS*	*AT2G34555 (AtGA2ox3)*	C04:65615366-65617201	317	35.84	8.38	Cytoplasm
BnaGA2ox4a	*BnaA05G0185600ZS*	*AT1G47990 (AtGA2ox4)*	A05:13019148-13021186	325	36.21	8.36	Cytoplasm

(Continued)

Table 1 (continued)

Gene name	Gene ID	Orthologues in *A. thaliana*	Chromosome location	Number of amino acids	Molecular weights (Mw, kDa)	Isoelectric point (pI)	Subcellular localization
BnaGA2ox4b	*BnaC05G0315700ZS*	*AT1G47990 (AtGA2ox4)*	C05:29963502-29965743	325	36.17	8.36	Cytoplasm
BnaGA2ox6a	*BnaA08G0322200ZS*	*AT1G02400 (AtGA2ox6)*	A08:28322382-28324750	332	37.03	7.00	Cytoplasm
BnaGA2ox6b	*BnaA10G0011600ZS*	*AT1G02400 (AtGA2ox6)*	A10:626155-628529	329	36.57	6.10	Cytoplasm
BnaGA2ox6c	*BnaC04G0031400ZS*	*AT1G02400 (AtGA2ox6)*	C04:3005510-3008368	329	36.72	6.40	Cytoplasm
BnaGA2ox6d	*BnaC05G0013500ZS*	*AT1G02400 (AtGA2ox6)*	C05:931557-933969	329	36.47	6.26	Cytoplasm
BnaGA2ox7a	*BnaA05G0174600ZS*	*AT1G50960 (AtGA2ox7)*	A05:11695710-11697806	332	38.12	8.43	Cytoplasm
BnaGA2ox7b	*BnaC05G0293500ZS*	*AT1G50960 (AtGA2ox7)*	C05:25841018-25844078	213	24.63	8.98	Cytoplasm
BnaGA2ox8a	*BnaA01G0114200ZS*	*AT4G21200 (AtGA2ox8)*	A01:6624652-6628779	338	38.97	5.96	Cytoplasm
BnaGA2ox8b	*BnaA03G0460000ZS*	*AT4G21200 (AtGA2ox8)*	A03:25077793-25080276	338	39.23	6.96	Cytoplasm
BnaGA2ox8c	*BnaC01G0139200ZS*	*AT4G21200 (AtGA2ox8)*	C01:9745003-9750935	337	38.89	5.66	Cytoplasm
BnaGA2ox8d	*BnaC03G0723400ZS*	*AT4G21200 (AtGA2ox8)*	C03:69767533-69776488	338	38.91	8.30	Cytoplasm
BnaGA2ox8e	*BnaC07G0436000ZS*	*AT4G21200 (AtGA2ox8)*	C07:54005227-54008013	338	39.21	6.96	Cytoplasm
BnaGA2ox9a	*BnaA02G0109700ZS*	*AT5G58660 (AtGA2ox9)*	A02:5823648-5826106	348	39.22	5.12	Cytoplasm
BnaGA2ox9b	*BnaC02G0134200ZS*	*AT5G58660 (AtGA2ox9)*	C02:9515638-9517747	251	28.34	4.81	Cytoplasm
BnaGA2ox9c	*Bnascaffold0025G0010600ZS*	*AT5G58660 (AtGA2ox9)*	Bnascaffold0025: 961409-963906	379	42.47	4.80	Cytoplasm

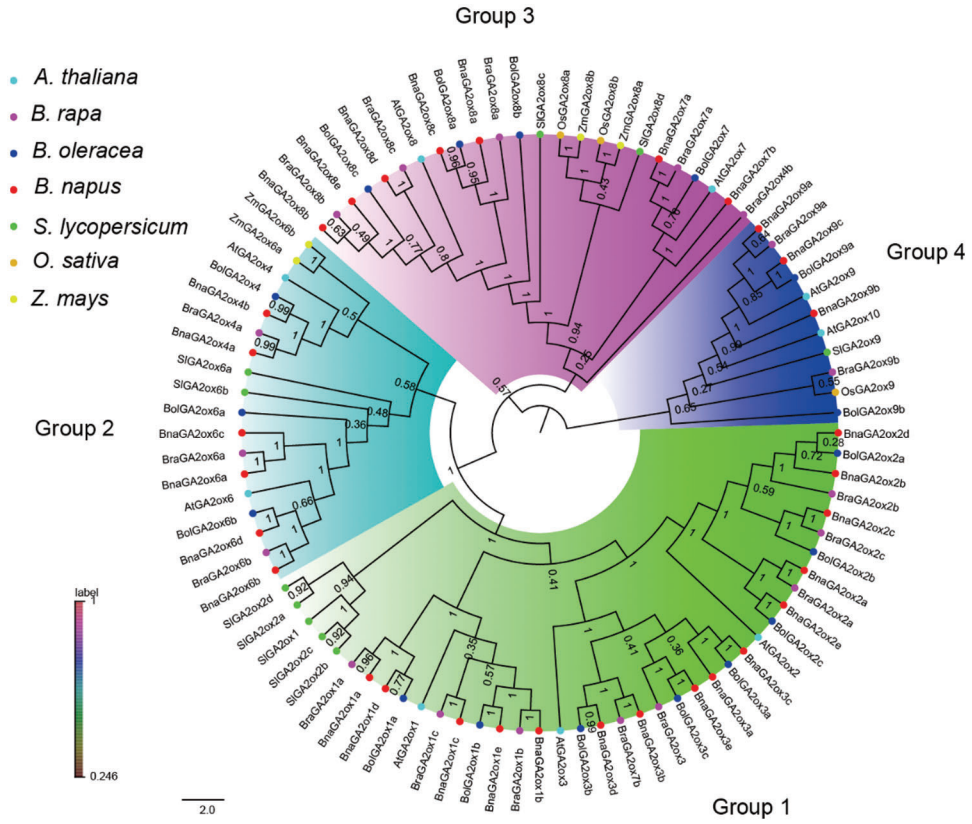

Figure 1: Phylogenetic relationship among GA2ox proteins in plants
Note: The NJ tree was generated with bootstrap analysis (1,000 replicates) using aligned GA2ox protein sequences from seven species and the tree was displayed with FigTree v1.4.0. At: *A. thaliana*; Bna: *B. napus*; Bra: *B. rapa*; Bol: *B. oleracea*; Sl: *S. lycopersicum*; Os: *O. sativa*; Zm; *Z. mays*.

3.3 Gene Structure and Conserved Motif Analysis of BnaGA2ox Family Members

To analyze and compare the intron/exon structures of *BnaGA2ox* family genes, their CDSs were aligned with the corresponding genomic sequences. The intron/exon structures among *BnaGA2ox* genes showed a wide difference, particularly the members belonging to different groups, while the *BnaGA2ox* genes of the same clade also showed certain exon segments and distributions (Fig. 2). All of the *BnaGA2ox* genes contained less exons and introns and none had more than four exons or introns. The single exon fragment in *BnaGA2ox* genes was less than one kilobyte. However, *BnaGA2ox8d* contained an intron of about eight kilobytes, *BnaGA2ox8c* had an intron of about five kilobytes, and *BnaGA2ox8a* contained an intron of about three kilobytes. The remaining *BnaGA2ox8a* genes had a single intron fragment smaller than two kilobytes.

To study protein structural diversity of BnaGA2ox members, the conserved motifs were analyzed by online MEME/MAST tools. We predicted ten putative protein motifs in BnaGA2ox proteins. Motif 1 and Motif 3 were detected in all 31 BnaGA2ox proteins (Fig. 3), and these motifs belonged to the 2OG-FeII_Oxy structural domain. Motif 2 was also present in all BnaGA2ox proteins, with the exception of BnaGA2ox9a protein, this indicates that the GA2ox family proteins are highly conserved in *B. napus*. BnaGA2ox proteins from different groups also contained some unique motifs. For example, Motif 10 was only detected in some BnaGA2ox proteins belonging to Group 2 and Group 3, and Motif 5 and Motif 6 were only present in the BnaGA2ox proteins belonging to Group 1 and Group 2. Motif 7 and Motif 8

were only detected in the BnaGA2ox proteins from Group 3 and Group 4. BnaGA2ox members from the same clade or subgroup showed a similar structural distribution, which was opposite to the gene structure distribution.

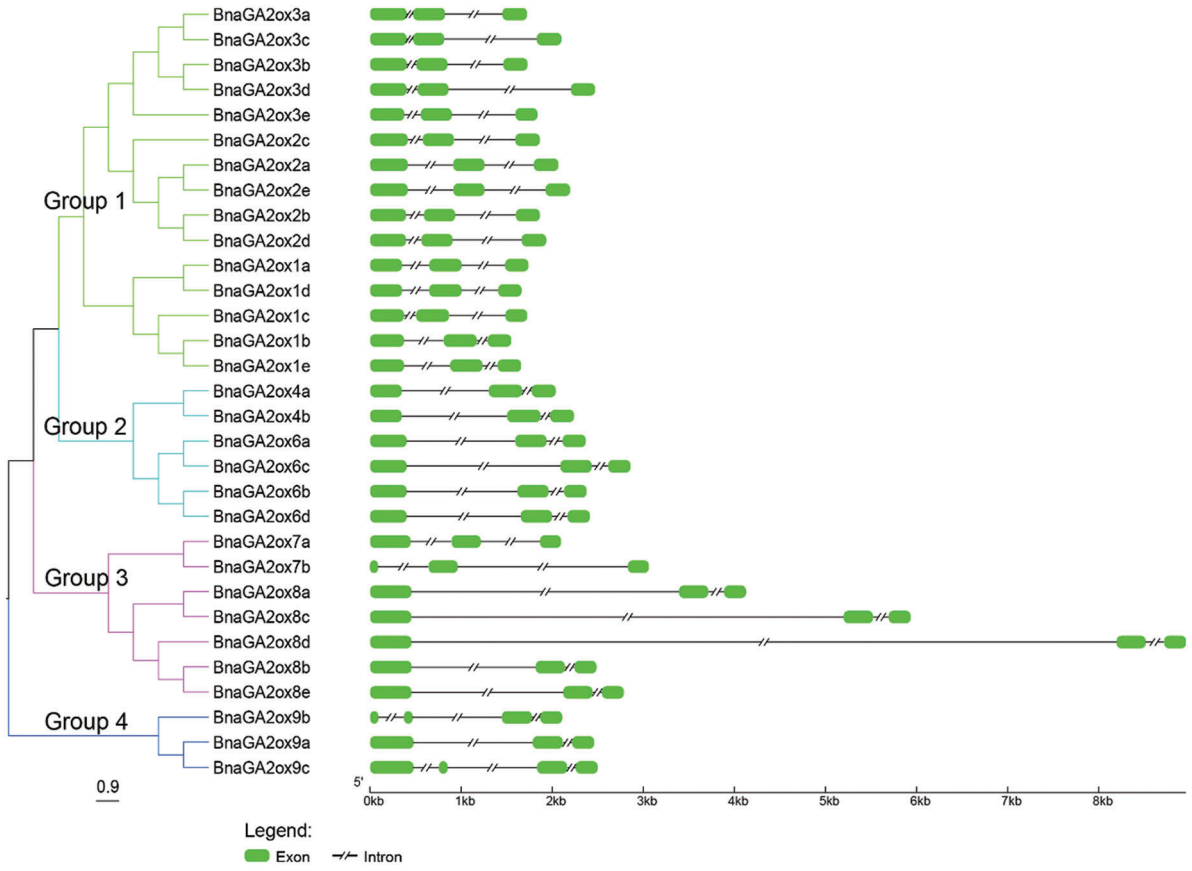

Figure 2: Phylogenic relationship and gene structure of *BnaGA2ox* genes
Note: The NJ tree was generated with bootstrap analysis (1,000 replicates) using aligned BnaGA2ox protein sequences, and the tree was displayed with FigTree v1.4.0.

3.4 Chromosomal Location and Syntenic Analysis of BnGA2ox Genes

To study chromosomal location of *BnaGA2ox* genes, we searched for their information in the published BnPIR database and visualized them on chromosomes using TBtools. A total of 30 *BnaGA2ox* genes were found on 14 chromosomes of *B. napus*, and one gene (*BnaGA2ox9c*) was on undefined Bnascaffold0025. Among them, 15 *BnaGA2ox* genes were located on the A and C subgenomes. No *BnaGA2ox* were found on chromosomes A04, A06, C08 and C09, and only one *BnaGA2ox* gene was found on each chromosome of A01, A09, C01 and C06 (Fig. 4). Two *BnaGA2ox* genes were identified on each of the A02, A03, A08, C02 and C07 chromosomes, and three *BnaGA2ox* genes were found on each of the A05, A07, C03, C04 and C05 chromosomes.

Through syntenic analysis of *GA2ox* genes between *B. napus* and *A. thaliana*, eight *AtGA2ox* genes showed a syntenic relationship with 25 *BnaGA2ox* genes (Fig. 4). Most of the syntenic *AtGA2ox* genes were located on chromosome A05, while the distribution of the syntenic *BnGA2ox* genes was relatively scattered. Among them, *AtGA2ox2* and *AtGA2ox8* corresponded to five syntenic *BnGA2ox* genes, and *AtGA2ox1* had four syntenic *BnGA2ox* genes, while the remaining *AtGA2ox2* showed two syntenic

BnGA2ox genes. We observed a tandem duplicated event between *BnaGA2ox1b* and *BnaGA2ox1c* in the *B. napus* genome.

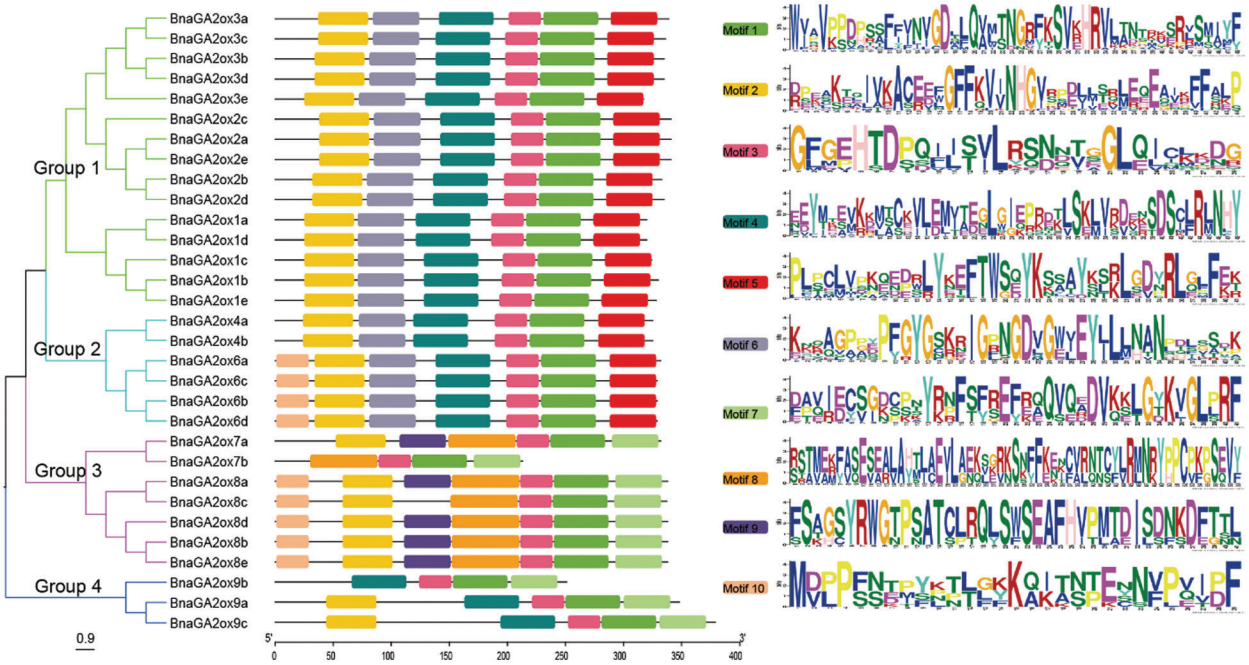

Figure 3: Phylogenic relationship and motif prediction of BnaGA2ox proteins
Note: The NJ tree was generated with bootstrap analysis (1,000 replicates) using aligned BnaGA2ox protein sequences and the tree is displayed with FigTree v1.4.0.

3.5 Selection Pressure Analysis

To study the selection pressure on *BnaGA2ox* genes, the *Ka*/*Ks* ratios were calculated between *BnaGA2ox* genes and its orthologous *AtGA2ox* genes, with *Ka*/*Ks* < 1 indicating purifying selection, *Ka*/*Ks* = 1 indicating neutral selection and *Ka*/*Ks* > 1 indicating positive selection [37]. The *Ka*/*Ks* ratios of *BnaGA2ox* genes ranged from 0.1066 (*BnaGA2ox6b*) to 0.3191 (*BnaGA2ox9c*) (Table 2). Comparing the *Ka*/*Ks* ratios between the A and C subgenomes, we found that the average *Ka*/*Ks* ratio of *BnaGA2ox* genes of the A subgenome was less than that of the C subgenome. This suggested that *BnaGA2ox* genes from the C subgenome underwent greater selection pressure during the evolution of *B. napus*. Moreover, we found that the average *Ka*/*Ks* ratio of *BnaGA2ox* genes of Group 4 was the highest, while the average *Ka*/*Ks* ratio of *BnaGA2ox* genes of Group 2 was the lowest among the four groups (Table 2). Among the *BnaGA2ox* genes belonging to Group 1, the *Ka*/*Ks* ratio of *BnaGA2ox1c* was lower than its homologous copies, and that of *BnaGA2ox2a* and *BnaGA2ox3a* were higher than their homologous copies. However, the *Ka*/*Ks* ratios of *BnaGA2ox* showed little difference among homologous copies in the other three groups, indicating that different evolutionary pressures may have been imposed to the *BnaGA2ox* homologous copies in Group 1 after the genome-wide triploidization of *B. napus*.

3.6 Putative Cis-Acting Elements in the Promoter Regions of BnaGA2ox Genes

To study and explore the potential transcriptional regulation of *BnaGA2ox* genes, the *cis*-acting elements were identified using the PlantCARE database. All *BnaGA2ox* genes contained the basic conserved functional motifs in their promoter regions, such as TATA-box and CAAT-box, as well as numerous important elements. To study the potential function of *BnaGA2ox* genes, we screened the *cis*-acting elements involved in light-responsive, biological or abiotic elements and phytohormones (Fig. 5). The

light response element was the most abundant. Most *BnaGA2ox* genes contained it in their promoter region, but it was mainly concentrated in the BnaGA2ox promoter region in Group 1. Two phytohormone *cis*-acting elements, the auxin (AUXRR-core/TGA-element) elements, were discovered in the promoter regions of *BnaGA2ox1a*, *BnaGA2ox1c*, *BnaGA2ox1d*, *BnaGA2ox8e*, *BnaGA2ox9a*, *BnaGA2ox9b* and *BnaGA2ox9c*. Gibberellin response elements, such as P-box, GARE-motif and TATC-box were found in the promoter regions of *BnaGA2ox4a*, *BnaGA2ox4b*, *BnaGA2ox6d*, *BnaGA2ox7a*, *BnaGA2ox8b*, *BnaGA2ox8e*, *BnaGA2ox9a*, *BnaGA2ox9b* and *BnaGA2ox9c*. Moreover, defense and abiotic stress, anaerobic induction and low temperature were detected in the promoter of *BnaGA2ox* genes in Groups 1 and 4. Drought-inducibility and MeJA and abscisic acid responsive elements were also examined in the promoter region of most *BnaGA2ox* genes. We also detected biological clock-related elements (circadian) in the promoter regions of *BnaGA2ox2b*, *BnaGA2ox2c* and *BnaGA2ox3c*.

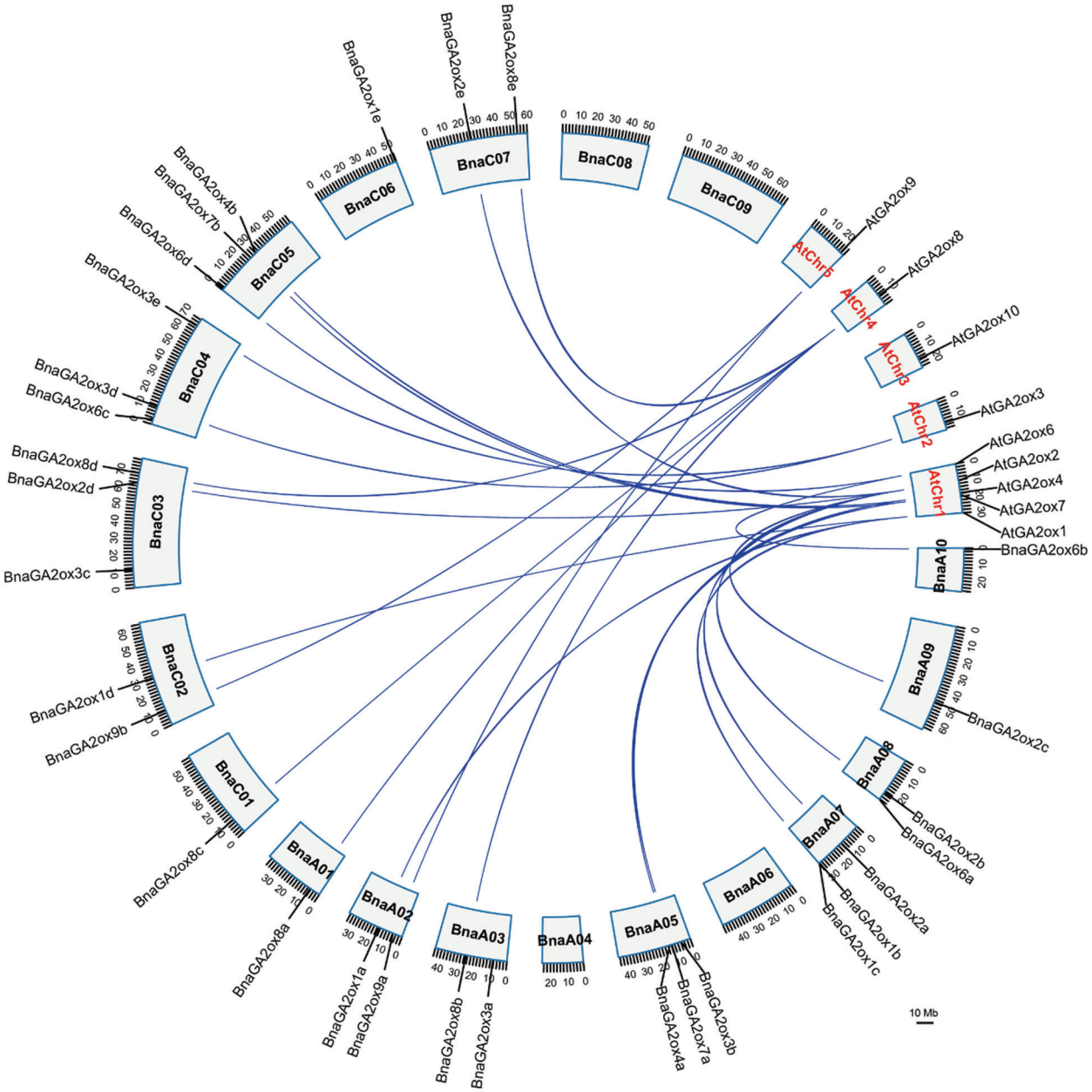

Figure 4: Chromosome location and syntenic relationship of *GA2ox* genes of *A. thaliana* and *B. napus*

Table 2: Non-synonymous and synonymous nucleotide substitution rates between *BnaGA2ox* and the corresponding *AtGA2ox* orthologs

Group	Gene name in *B. napus*	Gene name in *A. thaliana*	*Ka*	*Ks*	*Ka*/*Ks*	Average *Ka*/*Ks*	Subgenome
Group 1	*BnaGA2ox1a*	*AtGA2ox1*	0.0913	0.3527	0.2587	0.2319	A
	BnaGA2ox1b	*AtGA2ox1*	0.1273	0.4977	0.2557		A
	BnaGA2ox1c	*AtGA2ox1*	0.0888	0.4685	0.1896		A
	BnaGA2ox1d	*AtGA2ox1*	0.0950	0.3562	0.2665		C
	BnaGA2ox1e	*AtGA2ox1*	0.1128	0.4982	0.2265		C
	BnaGA2ox2a	*AtGA2ox2*	0.0672	0.3320	0.2024		A
	BnaGA2ox2b	*AtGA2ox2*	0.0525	0.4150	0.1266		A
	BnaGA2ox2c	*AtGA2ox2*	0.0636	0.3804	0.1671		A
	BnaGA2ox2d	*AtGA2ox2*	0.0508	0.4087	0.1244		C
	BnaGA2ox2e	*AtGA2ox2*	0.0620	0.3653	0.1697		C
	BnaGA2ox3a	*AtGA2ox3*	0.1249	0.5068	0.2465		A
	BnaGA2ox3b	*AtGA2ox3*	0.1158	0.3634	0.3186		A
	BnaGA2ox3c	*AtGA2ox3*	0.1310	0.4299	0.3047		C
	BnaGA2ox3d	*AtGA2ox3*	0.1082	0.3517	0.3078		C
	BnaGA2ox3e	*AtGA2ox3*	0.1147	0.3654	0.3141		C
Group 2	*BnaGA2ox4a*	*AtGA2ox4*	0.0663	0.3844	0.1725	0.1432	A
	BnaGA2ox4b	*AtGA2ox4*	0.0693	0.3866	0.1793		C
	BnaGA2ox6a	*AtGA2ox6*	0.0617	0.4444	0.1390		A
	BnaGA2ox6b	*AtGA2ox6*	0.0470	0.4412	0.1066		A
	BnaGA2ox6c	*AtGA2ox6*	0.0637	0.4394	0.1450		C
	BnaGA2ox6d	*AtGA2ox6*	0.0545	0.4717	0.1155		C
Group 3	*BnaGA2ox7a*	*AtGA2ox7*	0.1072	0.4170	0.2570	0.1860	A
	BnaGA2ox7b	*AtGA2ox7*	0.0906	0.4238	0.2139		C
	BnaGA2ox8a	*AtGA2ox8*	0.0659	0.3833	0.1720		A
	BnaGA2ox8b	*AtGA2ox8*	0.0457	0.2995	0.1527		A
	BnaGA2ox8c	*AtGA2ox8*	0.0690	0.3751	0.1840		C
	BnaGA2ox8d	*AtGA2ox8*	0.0612	0.3510	0.1742		C
	BnaGA2ox8e	*AtGA2ox8*	0.0440	0.2959	0.1485		C
Group 4	*BnaGA2ox9a*	*AtGA2ox9*	0.1093	0.3864	0.2828	0.3033	A
	BnaGA2ox9b	*AtGA2ox9*	0.2105	0.6834	0.3080		C
	BnaGA2ox9c	*AtGA2ox9*	0.1183	0.3707	0.3191		–

3.7 Gene Expression Pattern Analysis of BnaGA2ox Family Genes

To analyze and compare the gene expression levels of *BnaGA2ox* family genes, we downloaded their expression patterns in 90 tissues in different developmental stages from BnTIR. In *B. napus*, *BnaGA2ox*

genes showed low or no expression in the majority of tissues but some displayed tissue-specific expression (Fig. 6). Among them, *BnaGA2ox2b*, *BnaGA2ox2d*, *BnaGA2ox8a* and *BnaGA2ox8e* were highly expressed in roots, while the three copies of *BnaGA2ox8*, *BnaGA2ox8b*, *BnaGA2ox8c* and *BnaGA2ox8d* were highly expressed in stalks. *BnaGA2ox6d* was highly expressed in most detected leaves and *BnaGA2ox2c*, *BnaGA2ox6b* and *BnaGA2ox6d* were highly expressed in flowers. During silique development, *BnaGA2ox6d* highly expressed in the silique wall at all detected stages. *BnaGA2ox9a* and *BnaGA2ox9c* were consistently highly expressed in seeds at all detected stages. To verify the RNA-seq results, we evaluated the expression of four *BnaGA2ox* by qRT-PCR analysis, and the qRT-PCR results were in accordance with their RNA-seq results (Supplementary Fig. S2).

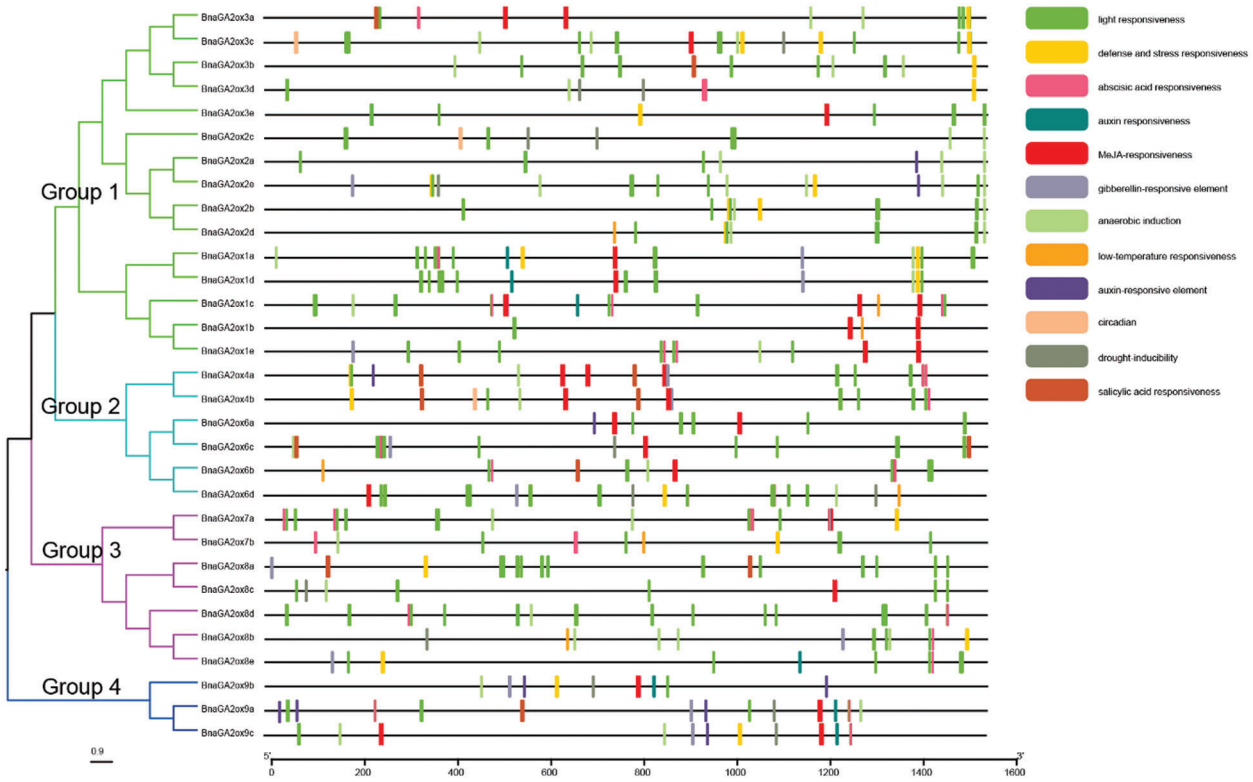

Figure 5: Genomic distribution of *cis*-acting elements in promoter regions of *BnaGA2ox* genes
Note: Different *cis*-acting elements are indicated by different colors. The *cis*-acting elements in promoter regions were predicted using PlantCARE.

3.8 Gene Expression Patterns of BnaGA2ox Family Genes under Phytohormone and Abiotic Treatment

To investigate the expression patterns of *BnaGA2ox* genes under phytohormone and abiotic treatments, their expressions at SLs and SRs were examined at various time points (Fig. 7). In SLs under phytohormone treatments, most of *BnaGA2ox* genes were not obviously induced by phytohormones, while *BnaGA2ox6d* were upregulated under aminocyclopropane carboxylic acid (ACC) treatment, and three *BnaGA2ox2* members, *BnaGA2ox2b*, *BnaGA2ox2c* and *BnaGA2ox2d*, were evidently induced by jasmonic acid (JA) (Fig. 7A). In SRs, most of the *BnaGA2ox* genes were unregulated or downregulated under phytohormone treatments (Fig. 7B). For example, *BnaGA2ox6d* were obviously unregulated under indoleacetic acid (IAA) and ACC treatments, while those were distinctly downregulated by gibberellin (GA), trans-Zeatin (TZ) and abscisic acid (ABA) treatments. Meanwhile, *BnaGA2ox2d* were also evidently induced by JA in root, and suppressed by GA and ACC, and *BnaGA2ox8e* were obviously induced by ACC and JA.

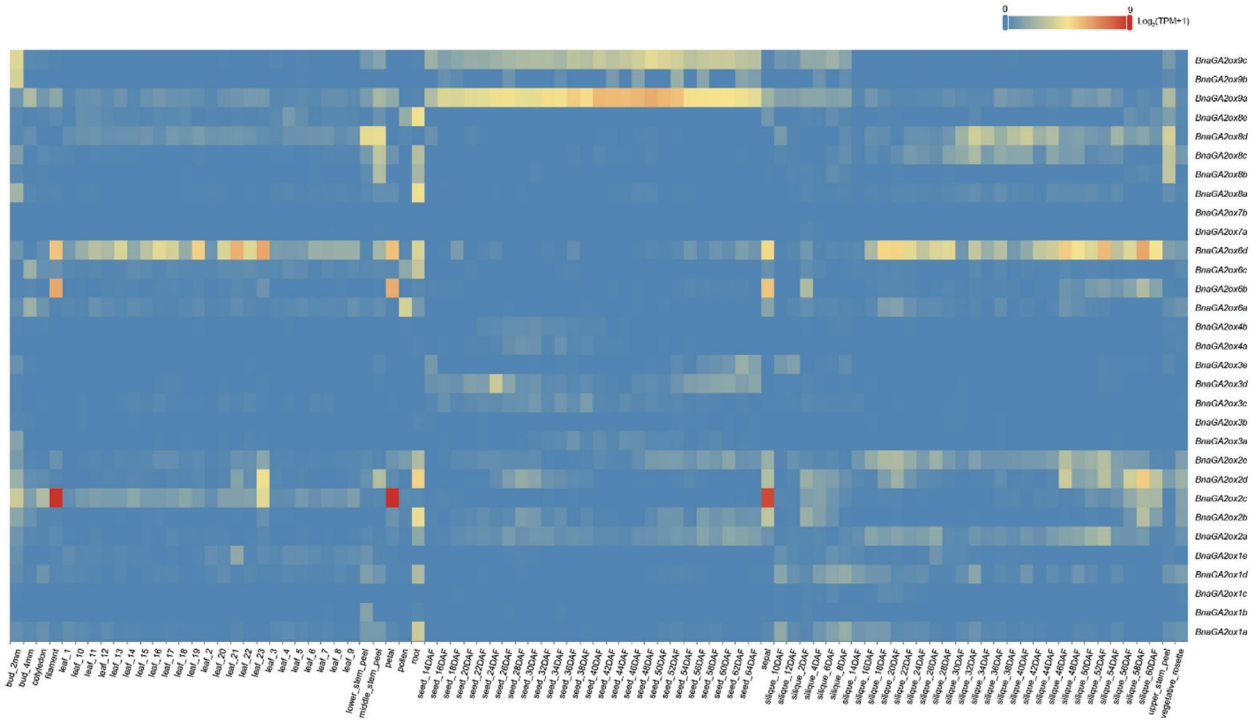

Figure 6: Expression patterns of *BnaGA2ox* family genes
Note: The color bar inthe figure represents the $\log_2$ expression values, and the expression values were normalized using TPM+1.

Under abiotic treatments, *BnaGA2ox6d* were found to be obviously upregulated by freezing, cold and osmotic treatments in SLs, but that was obviously upregulated by salt, drought, freezing and osmotic treatment in SRs (Figs. 7C, 7D). In SLs, we also discovered that *BnaGA2ox1d*, *BnaGA2ox2a*, *BnaGA2ox2e*, *BnaGA2ox2b* and *BnaGA2ox6c* were upregulated under cold treatment, and *BnaGA2ox1e* and *BnaGA2ox2d* were upregulated under drought treatment. In SRs, *BnaGA2ox2b*, *BnaGA2ox2d*, *BnaGA2ox2e* and *BnaGA2ox8e* were also found to be distinctly upregulated under drought treatment, and *BnaGA2ox8a* was upregulated by freezing induction.

4 Discussion

GAs play pivotal roles in plant development and growth. Decreasing and increasing levels of active GAs can both result in dwarf or semi-dwarf phenotypes. As key enzymes regulating GAs activity in plants, *GA2ox* family genes have been identified in multiple species including *A. thaliana* [11,18], *O. sativa* [16,17] and *Z. mays* [19]. We identified 31 *BnaGA2ox* genes in *B. napus*, as well as 18 *BraGA2ox* and 17 *BolGA2ox* genes, respectively, in its two parental species, *B. oleracea* and *B. rapa*. Consistent with previous studies [20], *GA2ox* genes had fewer exons in *B. napus* and showed similar protein motif distributions among homologous copies in each subfamily. This was consistent with previous studies on GA oxidase [38]. Most *cis*-acting elements were also detected in the promoter regions of multiple *BnaGA2ox* genes, including some specific *cis*-acting elements, such as defense and abiotic stress, low temperature, and drought-inducibility. This suggests that *BnaGA2ox* genes not only play important functions in plant development and growth, but also have potential functions in response to biotic or abiotic stresses, as well as some specific signals.

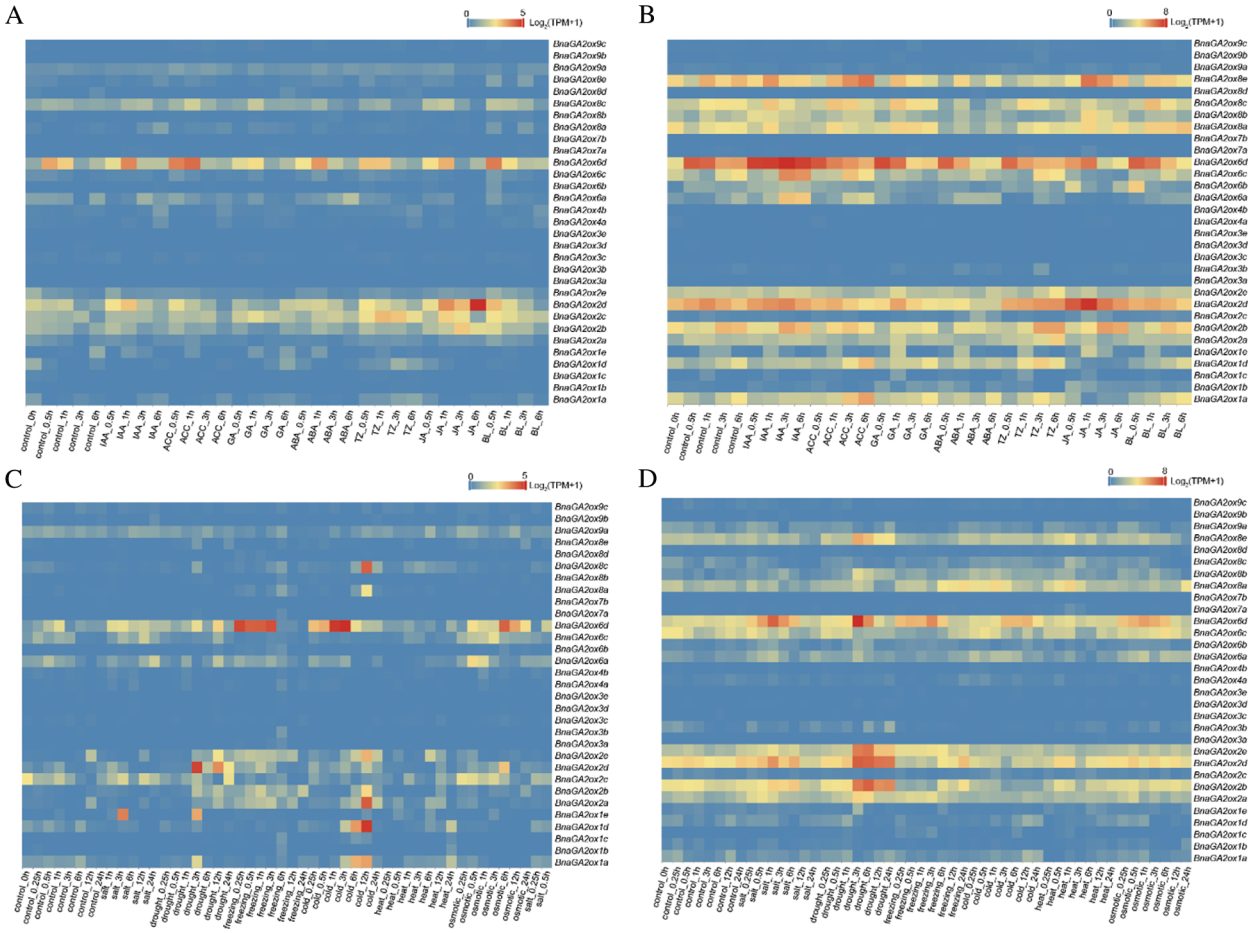

Figure 7: Expression patterns of *BnaGA2ox* family genes under phytohormone and abiotic treatments. (A) Expression patterns of *BnaGA2ox* family genes under phytohormone treatment in SLs. (B) Expression patterns of *BnaGA2ox* family genes under phytohormone treatment in SRs. (C) Expression patterns of *BnaGA2ox* family genes under abiotic treatment in SLs. (D) Expression patterns of *BnaGA2ox* family genes under abiotic treatment in SRs

Note: The color bar in the figure represents the $\log_2$ expression values, and the expression values were normalized using TPM+1.

Compared to the genomes of *O. sativa* and *Z. mays*, *Brassica* species experienced an extra genome-wide triploidization event in their genomes during their evolution [27,28]. However, there was no obvious difference among *B. rapa*, *B. oleracea*, *O. sativa* and *Z. mays* when comparing the gene number of *GA2ox*. Homology analysis found that three genes, *GA2ox1*, *GA2ox6* and *GA2ox8*, from *O. sativa* and *Z. mays* had multiple homologous copies through comparison with their homologous genes *AtGA2ox* (Supplementary Table S1). Previous studies revealed that polyploidization was the main force promoting the appearance of *OsGA2ox* copies during evolution [39]. Although numerous copy genes have been lost by diploidization after experiencing the tetraploid stage in the evolution of maize, approximately 30% of the genes retain multiple copies in the maize genome [40,41]. Certain *GA2ox* genes have two copies in the maize genome [19]. *GA2ox* genes can be divided into three groups in monocotyledon and dicotyledon plants [19,42], including two C_{19}-GA2ox Classes and one C_{20}-GA2ox Class. In this study, we added one new C_{20}-GA2ox Class, which was recently identified in *A. thaliana* and includes *AtGA2ox9* (*At5g58660*) and *AtGA2ox10* (*At3g47190*) [43], as well as their homologs identified in *B. rapa*, *B. oleracea*, *B. napus*,

O. sativa and *Z. mays*, *S. lycopersicum*. Phylogeny evidence indicates that *GA2ox* genes can be classified into four taxa, two C_{20}-GA2ox Classes and two C_{19}-GA2ox Classes. Among them, *GA2ox* genes are in an expansion state in Group 1 of C_{20}-GA2ox Class and Group 3 of C_{19}-GA2ox Class.

The expression patterns of *GA2ox* genes have been detected in several plants. In *A. thaliana*, the expressions of seven *AtGA2ox* genes suggest strong tissue-specific expression of *GA2ox* genes [44]. The tissue-specific expressions of *AtGA2ox2* and *AtGA2ox4* verified their importance in maintaining the levels of bioactive GAs in shoot apical meristem [45,46]. Consistent with their results, similar findings were found in *O. sativa* [47]. In *S. lycopersicum*, *SlGA2ox2* was expressed in flowers, roots, stems, leaves and immature fruits [48,49]. In *Z. mays*, the *GA2ox* genes are highly expressed in the shoot apical meristem and primary roots [19]. In this study, we found that some *BnaGA2ox* genes also had strong tissue-specific expression. For example, *BnaGA2ox2c*, *BnaGA2ox6b* and *BnaGA2ox6d* had high expression levels in sepals, petals and filaments of floral organs, and *BnaGA2ox9a* and *BnaGA2ox9c* were highly expressed in seeds at all detected stages, especially at 42–52 days after flowering. These data suggest that the balance of bioactive GAs and their intermediates, which are maintained through GA2ox enzymes, may play a crucial role in the precise regulation of plant growth. However, most of *BnaGA2ox* genes had no or low expression levels in almost all studied tissues. Consistent with the *cis*-elements analysis, we found that several *BnaGA2ox* genes were obviously induced under phytohormone or abiotic treatments, such as *BnaGA2ox6b* which was markedly induced by ACC, IAA, GA, freezing, cold, salt and drought. However, there was a wide difference in induction of phytohormone or abiotic treatments at SRs and SLs, and most of *BnaGA2ox* genes are more easily induced or suppressed at SRs under phytohormone or abiotic treatments. Moreover, most of *BnaGA2ox* genes were not induced by GA, and some of them were suppressed under GA treatment in SRs, suggesting that plants need to reduce the GA synthesis to maintain a dynamic balance of bioactive GAs in shoot apical meristems when spraying exogenous GAs [46]. The response to other phytohormones on *BnaGA2ox* genes indicates mutual regulation between GAs and other phytohormones. Surprisingly, we found that the light response of relative *cis*-elements occurs in most GA2ox promoter regions, but the expression results found that only *BnaGA2ox6b* was highly expressed in the petiolate leaves and silique pericarps of *B. napus*. Although we can not currently verify whether the expression of *BnaGA2ox* is induced by lighting, while previous studies have suggested that silique pericarps have replaced leaves as the main photosynthetic organs during the middle and late development of siliques in *B. napus* [50,51], indicating that *BnaGA2ox6b* may play a key role in *B. napus* photosynthesis.

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Authorship: The authors confirm contribution to the paper as follows: YZ and TH conceived and designed the experiments; YL, HH, YS, SH, PZ and XD performed the sampling and experiments; YL, TL, CX, XT and YZ contributed to data analysis and interpretation; YL and HH wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Supplementary Materials: Supplementary materials can be found at the end on Figs. S1 and S2 and Tables S1 and S2.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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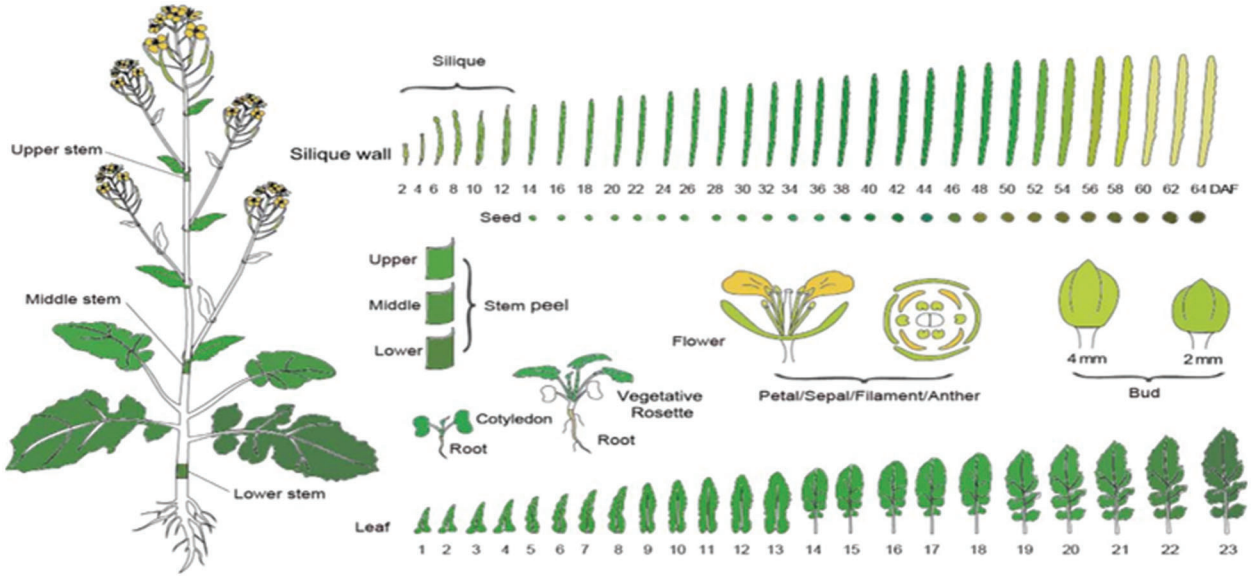
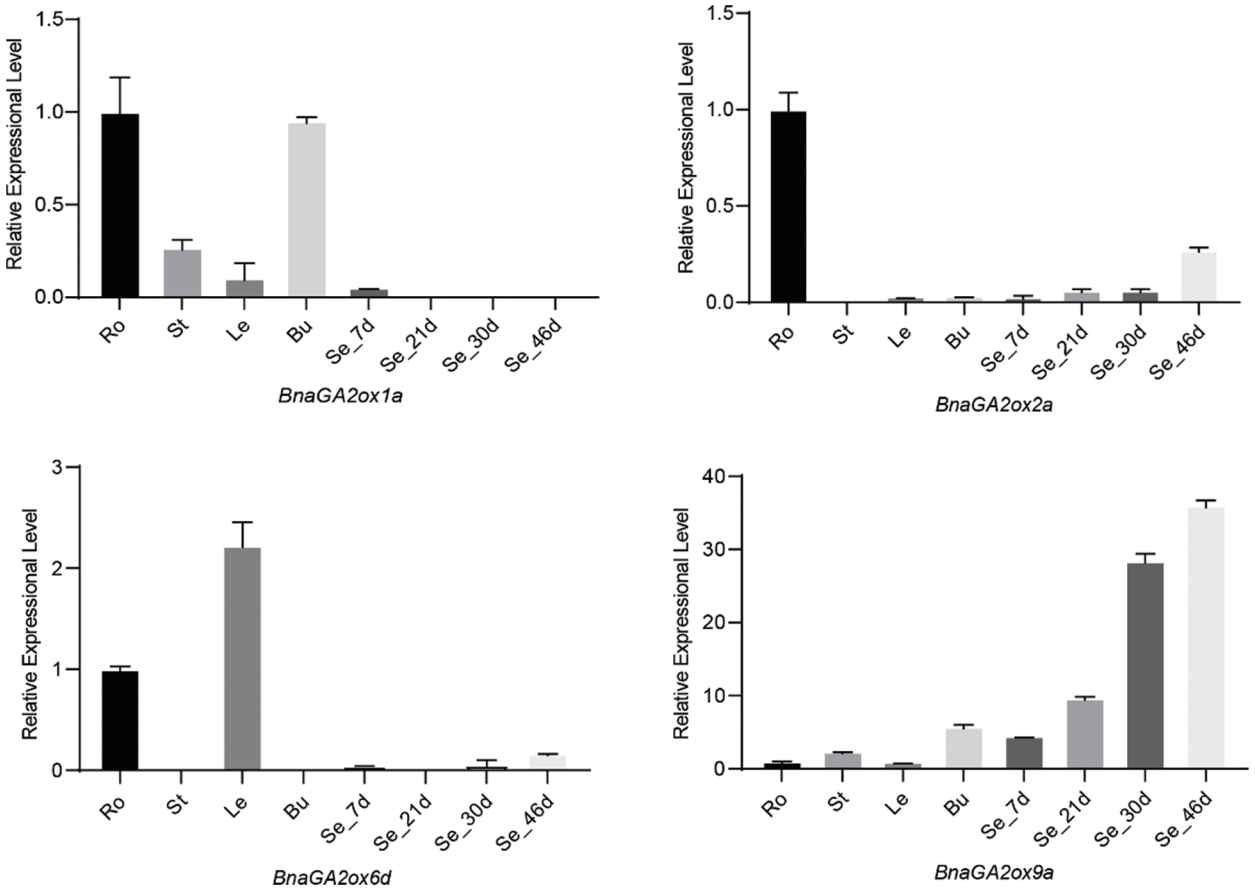
Appendix



Supplementary Figure S1: The diagram of 90 tissues in *B. napus*. The Figure downloaded from BnTIR database



Supplementary Figure S2: The qRT-PCR results for four selected genes

Supplementary Table S1: Primers used for qRT-PCR

	Sequence of forward primer (5′-3′)	Sequence of reverse primer (5′-3′)
BnaGA2ox1a	GGTTGAGTACTTGCTGATGAAC	CTGACAATGCGTTTCTGAAGAT
BnaGA2ox2a	AAACATGGTGGTTTTATCACGG	CTCACAAGCTTTCACGATAAGG
BnaGA2ox6d	CTCGTCTCGAGTTCTTCAAAAC	ACGTACACATGGTGTCTACTTT
BnaGA2ox9a	TGGACAAGGAGATACTAACAGAGG	CCTTCCATATTCCTCTATCAACAC
BnaACT7	TGGGTTTGCTGGTGACGAT	TGCCTAGGACGACCAACAATACT

Supplementary Table S2: The *GA2ox* genes in *B. rapa*, *B. oleracea*, *S. lycopersicum*, *O. sativa* and *Z. mays*

Gene ID in *B. rapa*	Gene Name in *B. rapa*	Gene ID in *B. oleracea*	Gene Name in *B. oleracea*	Gene ID in *S. lycopersicum*	Gene Name in *S. lycopersicum*	Gene ID in *O. sativa*	Gene Name in *O. sativa*	Gene ID in *Z. mays*	Gene Name in *Z. mays*
BraA02g024710.3C	*BraGA2ox1a*	*BolC02g034570.2J*	*BolGA2ox1a*	*Solyc07g056670.2.1*	*SlGA2ox1*	*XP_015637578.2*	*OsGA2ox6a*	*NP_001152057.1*	*ZmGA2ox6a*
BraA07g041560.3C	*BraGA2ox1b*	*BolC06g049410.2J*	*BolGA2ox1b*	*Solyc02g070430.2.1*	*SlGA2ox2a*	*XP_015639483.1*	*OsGA2ox6b*	*XP_008662690.1*	*ZmGA2ox6b*
BraA07g041570.3C	*BraGA2ox1c*	*BolC03g071130.2J*	*BolGA2ox2a*	*Solyc07g061720.2.1*	*SlGA2ox2b*	*XP_015649346.1*	*OsGA2ox2*	*XP_008675855.1*	*ZmGA2ox6c*
BraA07g010700.3C	*BraGA2ox2a*	*BolC05g027570.2J*	*BolGA2ox2b*	*Solyc07g061730.2.1*	*SlGA2ox2c*	*XP_015633380.1*	*OsGA2ox1a*	*NP_001131206.1*	*ZmGA2ox1a*
BraA08g023600.3C	*BraGA2ox2b*	*BolC07g015570.2J*	*BolGA2ox2c*	*Solyc08g016660.1.1*	*SlGA2ox2d*	*XP_015638414.1*	*OsGA2ox1b*	*NP_001148268.2*	*ZmGA2ox1b*
BraA09g034980.3C	*BraGA2ox2c*	*BolC03g020320.2J*	*BolGA2ox3a*	*Solyc05g053340.2.1*	*SlGA2ox6a*	*XP_015638821.1*	*OsGA2ox1c*	*NP_001348171.1*	*ZmGA2ox1c*
BraA04g024720.3C	*BraGA2ox3*	*BolC04g014580.2J*	*BolGA2ox3b*	*Solyc01g079200.2.1*	*SlGA2ox6b*	*XP_015624176.1*	*OsGA2ox8a*	*NP_001354056.1*	*ZmGA2ox1d*
BraA05g020440.3C	*BraGA2ox4*	*BolC04g060100.2J*	*BolGA2ox3c*	*Solyc02g080120.1.1*	*SlGA2ox8c*	*XP_015635159.1*	*OsGA2ox8b*	*XP_008657216.3*	*ZmGA2ox1e*
BraA08g035680.3C	*BraGA2ox6a*	*BolC05g034760.2J*	*BolGA2ox4*	*Solyc04g008670.1.1*	*SlGA2ox8d*	*XP_015645542.1*	*OsGA2ox8c*	*NP_001148252.2*	*ZmGA2ox8a*
BraA10g001200.3C	*BraGA2ox6b*	*BolC04g003710.2J*	*BolGA2ox6a*	*Solyc06g082030.2.1*	*SlGA2ox9*	*XP_015645543.1*	*OsGA2ox8d*	*XP_008645957.2*	*ZmGA2ox8b*
BraA05g018620.3C	*BraGA2ox7a*	*BolC05g001160.2J*	*BolGA2ox6b*			*XP_015645546.1*	*OsGA2ox8e*	*XP_008645958.2*	*ZmGA2ox8c*
BraA01g011970.3C	*BraGA2ox8a*	*BolC05g032070.2J*	*BolGA2ox7*			*XP_015634049.2*	*OsGA2ox9*	*XP_008651905.1*	*ZmGA2ox8d*
BraA03g049490.3C	*BraGA2ox8b*	*BolC01g014420.2J*	*BolGA2ox8a*					*XP_020396999.1*	*ZmGA2ox8e*
BraA08g014480.3C	*BraGA2ox8c*	*BolC03g077460.2J*	*BolGA2ox8b*					*XP_020397000.1*	*ZmGA2ox8f*
BraA02g011510.3C	*BraGA2ox9a*	*BolC07g049100.2J*	*BolGA2ox8c*					*XP_020397001.1*	*ZmGA2ox8g*
BraA10g016550.3C	*BraGA2ox9b*	*BolC02g014100.2J*	*BolGA2ox9a*					*XP_020395338.1*	*ZmGA2ox9*
BraA03g038630.3C	*BraGA2ox10*	*BolC09g047390.2J*	*BolGA2ox9b*						