Comprehensive In Silico Characterization and Expression Pro-Filing of DA1/DAR Family Genes in *Brassica rapa*

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Abstract: The DA1/DAR family genes have been shown to play important roles in regulating organ size and plant biomass in the model plant Arabidopsis and several crops. However, this family has not been characterized in *Brassica rapa* (*B. rapa*). In this study, we identified 17 DA1&DAR genes from *B. rapa*. Phylogenetic analysis indicated that these genes are classified into four groups. Structural and motif analysis of *BrDA1*&*DARs* discovered that the genes within the same group have similar exon-intron structures and share an equal number of conserved motifs except for *BrDAR6.3* from group IV, which contains two conserved motifs. *Cis*-regulatory elements identified four phytohormones (Salicylic acid, Abscisic acid, Gibberellin, and auxin) and three major abiotic (Light, Low temperature, and drought) responsive elements. Further, six br-miRNAs named br-miR164a, br-miR164b, br-miR164c, br-miR164d, br-miRN360, and br-miRN366 were found which target *BrDA1.5*, *BrDA1.4*, and *BrDA1.5*. *BrDA1*&*DAR* genes were highly expressed in stem, root, siliques, flower, leaf, and callus tissues. Moreover, qRT-PCR analyses indicated that some of these genes were responsive to abiotic stresses or phytohormone treatments. Our findings provide a foundation for further genetic and physiological studies of *BrDA1*&*DARs* in *B. rapa*.

Keywords: *Brassica rapa*; genome-wide; DA1&DAR family genes; expression profiling

1. Introduction

The DA1/DAR gene family plays important roles in regulating organ size and plant biomass. DA1 was first identified in the model plant *Arabidopsis thaliana* (*A. thaliana*) as a key regulator for organ size [1]. In the da1-1 mutant, the conversion of an amino acid from arginine to lysine at position 358 (*DA1*R358K) resulted in large leaves, flowers, siliques, and seeds due to an extended period of cell proliferation [1]. DA1 protein consists of two ubiquitin-interacting motifs (UIM), a zinc-binding LIM domain, and a C-terminal peptidase domain [1–3]. The Arabidopsis genome encodes seven DA1-related (DAR) proteins, among which DAR1 and DAR2 are the most closely related to DA1 based on amino acid sequences [1,4]. DAR1 acts redundantly with DA1 to regulate cell proliferation [1], while DA1, DAR1, and DAR2 redundantly regulate endoreduplication [4]. Recent studies have shown that the peptidase domain of DA1 and DAR proteins is important for its function on organ size regulation, and the peptidase activity is regulated by specific E3 ubiquitin ligases, de-ubiquitination enzymes, and receptor-like kinase [3].

In crops, the DA1/DAR family genes also function as key organ size and crop yield regulators. For instance, overexpression of the mutant version of *ZmDA1* or *ZmDAR1* enhances the starch synthesis and improves kernel yield in maize [5]. In wheat (*Triticum aestivum* L.), the expression level of *TaDA1* was correlated with kernel weight and yield [6]. In addition, BnDA1 is also associated with seed weight in *Brassica napus* (*B. napus*) [7]; down-regulation of BnDA1 improves the seed weight and organ size [7]. These studies suggested that the function of DA1 family genes in regulating organ size is highly conserved in different species and may be used as a molecular target for high-yield breeding.
Since the DA1&DAR gene family plays regulatory roles in developmental processes in plants, we wanted to identify BrDA1&DAR genes in B. rapa and investigate their involvement in different stress conditions. B. rapa is a diverse plant species belonging to Cruciferae (Brassicaceae) family, which is further divided into three well-defined groups named oil-type rape, leafy-type B. rapa, and rapiferous-type [8]. There have been no studies about the characterization and stress response behavior of the DA1&DAR gene family in B. rapa. We conducted a comprehensive genome-wide study to identify the DA1&DAR gene family in B. rapa. We identified 17 DA1&DAR genes in the B. rapa genome and analyzed the phylogenetic relationships, synteny examination, gene structures, conserved motifs, cis-elements in the promoter region, and miRNA regulator prediction. Additionally, we studied DA1&DAR gene expression in various tissues and under different abiotic and phytohormone treatments. These analyses are helpful for further functional characterization of the DA1&DAR gene family in B. rapa.

2. Materials and Methods

2.1. Identification and Characterization of DA1&DARs in B. rapa

Protein sequences of all BrDA1&DARs were obtained via accessing the Brassica database (http://www.brassicadb.cn/ (Version 3, 3.1 and 3.5), accessed on 10 June 2022). The amino acid sequences of AtDA1&DARs were downloaded from the A. thaliana database (http://www.arabidopsis.org/; accessed on 10 June 2022). Peptide sequences of AtDA1&DARs were used as query sequences to identify all BrDA1&DARs. Then (http://pfam.xfam.org/; accessed on 10 June 2022) was accessed to scrutinize the DA1-like domain (PF12315) in all sequences and verify that all recognized genes from the Brassica database belong to the BrDA1&DAR gene family. PROTPARAM on ExPASy (http://web.expasy.org/protparam/; accessed on 10 June 2022) was accessed to measure chemical and physical properties such as isoelectric points (PI) and molecular weight (MW). To predict the sub-cellular localization of BrDA1&DAR genes WoLF PSORT server (https://wolfpsort.hgc.jp/; accessed on 10 June 2022) was accessed. Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/; accessed on 10 June 2022) was used to analyze the gene structure of BrDA1&DAR genes. To identify the conserved motifs, MEME (V 4.11.4) was used.

2.2. Analysis of Phylogenetic Trees and Syntenic Pairing BrDA1&DAR Family Proteins

To observe and analyze the evolutionary background, the DA1&DAR family proteins of B. rapa, A. thaliana, B. napus, and B. oleracea were used to construct the phylogenetic tree. Alignment of protein sequences was performed using MEGA X (V6.06) software. The tree was constructed via the neighbor-joining (NJ) method with 1000 bootstrap replicates. Synteny associations of DA1&DAR genes between B. rapa, A. thaliana, B. oleracea, and B. napus were identified and analyzed via TBtools software V1.098; (https://github.com/CJ-Chen/TBtools, accessed on 10 June 2022).

2.3. Identification and Analysis Cis-Elements in the BrDA1&DAR Gene Promoters

Next, 2000 base pairs upstream of BrDA1&DAR genes were downloaded from the Brassica database (http://www.brassicadb.cn/; accessed on 10 June 2022) to find the cis-regulatory element. PlantCARE webtool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; accessed on 10 June 2022) was accessed to predict the cis-elements, then results were presented using TBtools software V 1.098; (https://github.com/CJ-Chen/TBtools, accessed on 10 June 2022).

2.4. Prophecy of Putative miRNA Targeting BrDA1&DAR Genes

BrDA1&DAR gene sequences were used as candidate genes to identify predicted miRNAs via the psRNATarget database (http://plantgrn.noble.org/psRNATarget/; accessed on 10 June 2022) with default parameters. The interaction network between the predicted miRNAs and the target BrDA1&DAR genes was created using Cytoscape V3.8.2 software (https://cytoscape.org/; accessed on 10 June 2022).
2.5. Expression Profiling of BrDA1&DAR Genes in Various Tissues

We used RNA-seq data [9] to highlight the expression patterns of BrDA1&DAR genes. Expression was analyzed from six tissues (root, stem, leaf, callus, flower, and silique) of B. rapa accession Chiifu-401-42. Expression values were analyzed in FPKM (fragments per kilobase of exon model per million mapped reads). We identified and generated the heatmap of expression of BrDA1&DAR genes using GraphPad Prism 9.0.0 software (https://www.graphpad.com/, accessed on 10 June 2022).

2.6. Plant Material and Stress Conditions

Wild-type Chinese cabbage “A03” was grown under different stress conditions. Seeds with 100% germination were selected and the growing conditions were 25 °C Day/Night and 16 h light and 8 h dark cycle. The effects of phytohormones on the seedlings were determined by treating them with 100 µM gibberellic acid (GA), 100 µM abscisic acid (ABA), 100 µM salicylic acid (SA), and 100 µM indole-acetic acid/auxin (IAA). The samples were collected at successive 2 h after the application of stress, i.e., 0 h (CK as control), 2 h, 4 h, 6 h, 8 h. Polyethylene Glycol PEG6000 15% solution was applied for the imposition of drought stress and samples were collected at 0 h (CK), 2 h, 4 h, 6 h, 8 h. For salt stress, NaCl 250 mM solution was applied, and samples were collected at 0 h (CK), 6 h, 12 h, 18 h, 24 h after the treatment. Samples were collected at 4 °C for cold stress analysis, and for heat stress, the temperature was set at 44 °C. After the stress imposition, samples were collected at 0 h (CK), 1 h, 3 h, 6 h, 12 h, and 24 h. the leaves were immediately immersed in liquid nitrogen and kept stored at −80 °C until further analysis.

2.7. RNA Extraction and qRT-PCR Analysis

For RNA extraction, Eastep® Super RNA Isolation Kit, Promega Biotech, Shanghai, China was used according to the protocol provided by the manufacturer. Then the quantity of extracted RNA was measured on Nanodrop One (Thermo Fisher Scientific, Worcester, MA, USA). The cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) was used for cDNA synthesis. Afterward, deionized water was added to dilute the cDNA into a 10× solution. qRT-PCR was performed with a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA), using SYBER® Green Supermix (Bio-Rad). BrActin primers were used as a control to analyze the results. The qRT-PCR reaction was performed as follows: 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for the 30 s, and 72 °C for 10 s. Each reaction was performed with three biological replicates, and then all the results were analyzed by using the 2^−ΔΔCT method as described. All the primers used in this experiment are listed in Supplemental Table S6.

3. Results

3.1. Identification of BrDA1&DAR Gene Family in B. rapa

Using AtDA1&DAR protein sequences as a query, BLASTP identified 17 DA1&DAR genes in the complete genome of B. rapa (Table 1). These genes were named BrDA1.1-BrDA1.5, BrDAR1.1-BrDAR1.3, BrDAR1.4-BrDAR1.6, BrDAR2.1-BrDAR2.5, BrDAR3.1 and BrDAR6.1-BrDAR6.3. We submitted amino acid sequences of all the BrDA1&DARs to the NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, accessed on 10 June 2022). We then analyzed the results in TBtools software V 1.098 to confirm the presence of the DA1-like domain in all identified genes (Supplemental Figure S1). Furthermore, we accessed http://pfam.xfam.org/ to verify the presence of the DA1-like domain in all sequences. Gene length varies from 2208 bp (BrDA1.3) to 8063 bp (BrDAR1.2) with 9 and 13 exons, respectively, and the highest number of exons was found in BrDAR1.1, which is 22. The coding sequences (CDS) varied from 462 bp (BrDAR6.3) to 2929 bp (BrDAR1.2), while the protein size varied from 154 (BrDAR6.3) to 976 (BrDAR1.2). The molecular weight of all BrDA1 proteins ranged from 17.11 kDa (BrDAR6.3) to 110.31 (BrDAR1.2). The subcellular localization predicted that 13 BrDA1&DAR proteins are localized on the nucleus, two on the cytoplasm, one on the
endoplasmic reticulum, and one on the peroxisome. Meanwhile, 13 DA1&DAR family genes from *Brassica oleracea* (*B. oleracea*) and *B. napus* were also identified (Supplemental Table S1).

### 3.2. Phylogenetic Relationships of BrDA1&DAR Genes

To understand the evolution of BrDA1&DARs, AtDA1&DARs, BnDA1&DARs, and BolDA1&DARs, an unrooted phylogenetic tree was established that was further divided into four groups (Groups I–IV) (Figure 1). Detailed results showed Group I contained 12 DAR family members (5 BrDAR2, 4 BnDAR2, 2 BolDAR2, and 1 AtDAR2); Group II consisted of 6 DAR1 (3 BrDAR1, 1 BnDAR1, 1 BolDAR1, and 1 AtDAR1); Group III had 12 DA1 members (5 BrDA1, 4 BnDA1, 2 BolDA1, and 1 AtDA1) and Group IV comprised of 19 members including 4 DAR3, 1 DAR4, 3 DAR5, 10 DAR6, and 1 DAR7 gene. Notably, Groups I and III had more BrDA1&DAR genes than the other two groups. Furthermore, it was found that BrDA1&DAR genes are closer to BnDA1&DARs and BolDA1&DARs.

![Figure 1. Phylogenetic analysis of 17 DA1&DAR proteins from *B. rapa*, 11 from *B. napus*, 13 from *B. oleracea*, and 7 from *A. thaliana*.](image-url)
Table 1. Detailed information of 17 BrDA1&DAR gene family.

| Transcript ID      | ID in AT   | Name Found on Database | Given Name | Genomic Location | Gene/CDS Length (bp) | Protein Length (AA) | Protein Molecular Weight (kDa) | Isoelectric Point (pI) | No of Exon/intron | Predicted Sub-Cellular Localization |
|--------------------|------------|-------------------------|------------|------------------|----------------------|---------------------|---------------------------|---------------------|------------------|-------------------------------------|
| BraA06g014880.3C   | AT1G19270  | DA1                     | BrDA1.1    | A06:7,920,264−7,922,541+ | 2278/1584           | 528                 | 59.68                     | 5.89                | 9/8              | Nucleus                                           |
| BraA06g015110.3C   | AT1G19270  | DA1                     | BrDA1.2    | A06:7,919,363−7,922,691+ | 3329/1593           | 531                 | 59.93                     | 5.89                | 9/8              | Nucleus                                           |
| BraA06g015150.3C   | AT1G19270  | DA1                     | BrDA1.3    | A06:7,920,334−7,922,541+ | 2208/1428           | 476                 | 53.67                     | 6.38                | 9/8              | Nucleus                                           |
| BraA08g028280.3C   | AT1G19270  | DA1                     | BrDA1.4    | A08:19,778,813−19,781,054−   | 2242/1539           | 513                 | 58.56                     | 6.06                | 8/7              | Nucleus                                           |
| BraA08g028910.3C   | AT1G19270  | DA1                     | BrDA1.5    | A08:19,778,513−19,781,625−   | 3113/1536           | 512                 | 58.6                      | 6.06                | 8/7              | Nucleus                                           |
| BraA01g001960.3C   | AT4G36860  | DAR1                    | BrDAR1.1   | A01:9,92,491−9,96,203+       | 3713/1704           | 568                 | 64.53                     | 4.99                | 22/21            | Nucleus                                           |
| BraA01g001980.3C   | AT4G36860  | DAR1                    | BrDAR1.2   | A01:9,93,314−1,001,376+      | 8063/2929           | 976                 | 110.31                    | 5.22                | 11/10            | Nucleus                                           |
| BraA01g001960.1C   | AT4G36860  | DAR1                    | BrDAR1.3   | A01:9,93,037−9,95,992+       | 2956/1662           | 554                 | 62.89                     | 5.62                | 12/11            | Cytoplasm                                          |
| BraA05g006240.3C   | AT2G39830  | DAR2                    | BrDAR2.1   | A05:3,156,572−3,160,445+     | 3874/1545           | 515                 | 58.24                     | 8.55                | 8/7              | Nucleus                                           |
| BraA03g021030.3C   | AT2G39830  | DAR2                    | BrDAR2.2   | A03:10,048,165−10,054,720−   | 6556/1545           | 515                 | 57.94                     | 7.09                | 12/11            | Nucleus                                           |
| BraA05g006190.3C   | AT2G39830  | DAR2                    | BrDAR2.3   | A05:3,157,807−3,160,445+     | 2639/1218           | 406                 | 46.62                     | 7.79                | 12/11            | Nucleus                                           |
| BraA03g021060.3C   | AT2G39830  | DAR2                    | BrDAR2.4   | A03:10,048,359−10,051,673−   | 3315/1338           | 446                 | 50.69                     | 6.05                | 10/9             | Endoplasm reticulum                                |
| BraA05g006160.3C   | AT2G39830  | DAR2                    | BrDAR2.5   | A05:3,156,521−3,160,636+     | 4116/1320           | 440                 | 50.36                     | 8.36                | 9/8              | Nucleus                                           |
| BraA09g009490.3C   | AT5G66640  | DAR3                    | BrDAR3.1   | A09:5,407,581−5,409,900−     | 2320/1551           | 517                 | 59.32                     | 6.1                 | 9/8              | Peroxisome                                         |
| BraA07g017320.3C   | AT5G66620  | DAR6                    | BrDAR6.1   | A07:14,995,298−14,998,531−   | 3234/2520           | 840                 | 95.28                     | 5.04                | 10/9             | Nucleus                                           |
| BraA09g009700.3C   | AT5G66620  | DAR6                    | BrDAR6.2   | A09:5,411,650−5,414,348−     | 2699/1479           | 493                 | 56.6                      | 5.18                | 13/12            | Nucleus                                           |
| BraA09g009770.3C   | AT5G66620  | DAR6                    | BrDAR6.3   | A09:5,411,699−5,414,567−     | 2569/462            | 154                 | 17.11                     | 6.43                | 3/2             | Cytoplasm                                          |
3.3. Synteny Analysis of BrDA1&DAR Genes

Tandem and segmental duplication always support plant genome progression and the development of gene family members. The mechanism segmental and tandem duplication developments were examined in BrDA1&DAR genes. The distribution of 16 BrDA1&DAR genes on chromosomes was evaluated in which 6 out of 10 chromosomes contained BrDA1&DAR genes (Figure 2). Chromosomes A01, A05, A06, and A09 possess three BrDA1&DARs, while A03 and A08 have two genes. The remaining chromosome did not contain any DA1&DAR genes. However, no paralogous gene was found on any chromosome. All BrDA1&DAR genes were found as evolved through tandem duplication. No proximal duplication was found on any chromosomes (Figure 2). These findings showed that tandem duplication actions played an important role in the expansion of the DA1&DAR gene family in B. rapa.

Collinearity analysis was performed to discover orthologs of DA1&DARs between B. rapa, A. thaliana, B. oleracea, and B. napus (Figure 3). Briefly, In B. rapa, the sequences of 17 genes belonging to BrDA1&DARs were collected from the latest genome assemblies V3, 3.1, 3.5. However, there were only 6 BrDA1&DARs that showed syntenic relationships with 6 AtDA1&DARs, 7 BnDA1&DARs, and 12 BrDA1&DARs present in V3.0 (http://www.brassicadb.cn/, accessed on 10 June 2022). In detail, one AtDA1 from Chr1 is associated with BrDA1.1 gene of A06 and BrDA1.4 on A08, one AtDAR2 from Chr2 is related to BrDAR2.1 gene of A05, AtDAR1 from Chr4 is associated to one BrDAR1.2 gene on A01, and AtDAR6 gene from Chr5 made syntenic association with one BrDAR6.1 gene on A07 and BrDAR3.1 on A09. The syntenic relationship between the DA1&DAR family of B. rapa, B. oleracea, and B. napus is presented in Figure 3.
Ka, Ks, and Ka/Ks ratio was measured to study the evolutionary process of BrDA1&DAR genes, and less than 1 Ka/Ks ratio was found for all duplicated genes, suggesting that the DA1&DAR gene family has been facing the discriminatory burden and selection pressure throughout its evolutionary process (Supplemental Table S2).

3.4. Gene Structure and Conserved Motifs Analysis of BrDA1&DAR Gene Family

To enhance our understanding of gene development of the B. rapa DA1&DAR family, exon-intron configuration was observed. Introns of the BrDA1&DARs ranged from 2 to 21 (Table 1; Figure 4A). Group I has 7 to 11 introns while Group II has the highest 11 to 21, Group III has 7 to 8 introns, and Group IV has 2 to 12 introns. BrDAR1.1 from group II has the highest introns, while BrDAR6.3 from group IV has the lowest introns, only 2. Group I has 8 to 12 exons, Group II has the highest number of exons ranging from 12 to 22, Group III has 8 to 9 exons, and Group IV has 3 to 13 exons. Intron-exon patterns were similar between Groups I and III, whereas Groups II and IV exhibited different intron/exon associations. These results indicate that most of the same group members had identical gene structures, supporting their phylogenetic relations.
Figure 4. Structural and motif analysis of BrDA1&DAR genes family. According to the phylogenetic analysis, the BrDA1&DAR genes were classified into four groups. (A) Gene structure analysis. (B) The conserved motifs of BrDA1&DARs.

We also studied protein sequences of all 17 BrDA1&DARs through MEME to identify the preserved region and found that their conserved motif of BrDA1&DAR proteins varies from 2 to 8. Motif distributions within groups were similar, especially in Groups I, II and III (Figure 4B). Group II and III members have eight conserved motifs except for BrDAR1.3 from Group II, which has seven conserved regions. Conversed motifs in Group I ranged from 5 to 8, a single member from Group I has five conserved regions, and Group IV possesses 5 to 6 conserved motifs except for BrDAR6.3, which has only two conserved regions only (Supplemental Table S3). The group classification based on phylogenetic relationship, gene structure, and conserved region analysis strongly supported that BrDA1&DAR proteins have identical peptide remains and that most of the members of the same group have similar roles.

3.5. Cis-Elements in Promoters of BrDA1&DAR Genes

We searched 2000 bp regions from the transcriptional active site of each gene against the PlantCARE database to identify cis-elements in their promoter region (Supplemental Table S4). Among four different hormones responsive elements SA, ABA, and IAA-related elements were widely distributed among different BrDA1&DAR genes showing their unique and vital role in phytohormone-regulated plant development and stress responses (Figure 5; Supplemental Table S4). Besides this, four different stress-responsive regulatory elements, such as anaerobic, drought, light, and low temperature, were also identified, indicating the stress-responsiveness of these genes (Figure 5; Supplemental Table S4). Identification of hormones and stress-related cis-elements indicates that expression profiling of BrDA1&DAR genes may vary under hormonal and abiotic stress conditions.
Figure 5. Different abiotic stress (anaerobic, light, drought, low temperature) and phytohormone (SA, ABA, GA, IAA) related cis-regulatory elements in BrDA1&DAR. Boxes of different colors show the different identified elements.

3.6. Identification of miRNA Targeting Sites in BrDA1&DAR Genes

During the last few years of innovative research, many investigations have found that miRNA mediates regulations of different genes under some stress conditions. We found six putative miRNAs that target three BrDA1&DAR genes (Figure 6). The complete information is available (Figure 6, Supplemental Table S5). Among them, four belong to the br-miR164 family that targets the BrDAR6.1 gene, whereas br-miRN366 and br-miRN360 target BrDA1.4 and BrDA1.5 in B. rapa (Figure 6).

Figure 6. The miRNA targeting sites in BrDA1&DAR genes (BrDAR6.1, BrDA1.5, and BrDA1.4).
3.7. Expression Profiling of BrDA1&DAR Gene Family

To demonstrate the expression of BrDA1&DARs, we used the RNA-Seq data of six different tissues that were analyzed by Tong [9]. The six genes (BrDA1.1, BrDA1.4, BrDAR1.2, BrDAR2.1, BrDAR2.2, and BrDAR3.1) are from Version 3, which has undergone transcriptome analysis. Other genes are from the upgraded versions 3.1 and 3.5, for which no transcriptome or expression data is currently available. The transcript profiling of these six BrDA1&DAR genes from root, stem, leaf, callus, flower, and silique showed that BrDA1.1, BrDA1.4, and BrDAR1.2 were highly expressed in all tissues, especially in root, flower, silique, and callus, which reflected their significant role in growth processes of B. rapa (Figure 7).

![Figure 7. Heatmap presentation of BrDA1&DAR genes expression in various tissues (root, stem, leaf, callus, flower, and silique). The bar at the bottom presents the expression value.](image)

3.8. Expression Analysis of BrDA1&DAR Genes under Hormone Treatment and Abiotic Stresses

Upon hormone treatment, most of the genes showed relatively high expression in response to ABA, while most genes showed relatively low expression under SA, IAA, and GA (Figure 8). For instance, in response to ABA, BrDA1.1, and BrDA1.4 at 6 h, BrDAR1.1 and BrDA2.3 at 4 h, BrDA2.1 at 8 h, BrDA2.2, BrDA2.5, BrDA6.1 at 4 h and 6 h and BrDA3.1 at 6 h and 8 h were upregulated. Under salicylic acid (SA), BrDA1.1 at 4 h and 8 h, BrDA1.3 at 8 h, BrDAR2.4 and BrDAR2.5 at 2 h, BrDA6.2 and BrDAR6.3 at 2 and 8 h were found to be highly expressed. While BrDAR1.1, BrDAR1.2, BrDAR2.2, BrDAR6.1, and BrDAR6.2 showed high expression under IAA at different time intervals. In response to SA, only four genes (BrDA1.2, BrDA1.3, BrDAR6.2, and BrDAR6.3) were upregulated at different time points, as shown in Figure 8. However, the remaining BrDA1&DARs did not show any significant difference under phytohormone stress (Figure 8).
Figure 8. Expression profiling of BrDA1&DAR under different phytohormones (ABA, GA, SA, and IAA). 0 h, 2 h, 4 h, 6 h, and 8 h are the time intervals of sampling after treatment, and graphs represent relative gene expression examined through the $2^{-\Delta\Delta CT}$ method.

Interestingly, most genes showed varied expression levels under cold and high temperatures (Figure 9). BrDA1.3, BrDA1.5, and BrDAR3.1 were most upregulated under low temperature ($4 \, ^\circ \text{C}$) compared to others, while BrDA1.4, BrDAR2.1, BrDAR6.1, and BrDAR6.3 were highly expressed at high-temperature treatment ($44 \, ^\circ \text{C}$).
Figure 9. Relative expression patterns of BrDA1&DAR genes under cold (4 °C) and high temperature (44 °C) treatment. Time points of 0 h as CK, 1 h, 3 h, 6 h, 12 h, and 24 h displayed the sampling intervals. Relatively few BrDA1&DAR genes showed high expression under drought and salinity stress as compared to phytohormones and temperature stresses. In detail, BrDA1.3, BrDA1.5, BrDAR1.1, BrDAR1.2, BrDAR2.2, BrDAR2.3, BrDAR6.2, and BrDAR6.3 were upregulated at different time points under drought stress while remaining genes showed minimum expression (Figure 10a). Under salinity stress, BrDA1.2, BrDA1.3, BrDA1.5, Br-
DAR1.1, BrDAR2.2, BrDAR2.4, BrDAR6.2, and BrDAR6.3 were upregulated as compared to other genes (Figure 10b).

Figure 10. Cont.
Figure 10. (a) Expression profiling of BrDA1&DAR genes under drought stress. Sampling was performed at 0 h (CK), 2 h, 4 h, 6 h, and 8 h. $2^{-\Delta\Delta CT}$ method was used to examine the results. (b) Expression patterns of BrDA1&DAR genes under salinity stress. Sampling was performed at 0 h (CK), 6 h, 12 h, and 24 h. $2^{-\Delta\Delta CT}$ method was used to analyze the results.

4. Discussion

In this study, we identified 17 BrDA1&DAR proteins containing DA1-like domain [10,11]. Moreover, we identified 13 from B. oleracea and 11 from B. napus (Table 1; Supplemental Table S1). Whole genome sequencing plays a crucial role in developing a strong understanding of evo-
lution, domestication, and diversification, as demonstrated by whole-genome triplication (WGT) of Brassica species, such as B. rapa and B. oleracea [12]. Among Brassica plants, many species display extreme traits, and each one underwent a WGT event when compared with A. thaliana. Although many of the paralogous genes derived from the WGT event were fractionated (loss of a duplicate), the conserved ones probably play a significant role in the domestication and the diversification of phenotypic characters [13]. According to phylogenetic analysis, all DA1&DARs were classified into four different groups (Figure 1). Group I and III have more BrDA1&DARs, as both shared 5 genes, while Group II possesses three and Group IV contains 4 BrDA1&DARs (Figure 1). The presence of all DA1&DARs in four groups shows their strong phylogenetic relationship, implying their close evolutionary correlation (Figure 1). Further investigation of gene structure analysis also supports these results, as BrDA1&DAR genes from the same groups have a conserved number of introns (Figure 4). Furthermore, motif regions within the group classification were comparable (Figure 4B). Notably, BrDAR6.3 from group IV has a diverse motif pattern compared to other members, suggesting its unique role in physiological and molecular functions as a growth regulator (Figure 4B). Proteins with strong evolutionary relationships and sharing similar motif composition suggest quite a similar function of BrDA1&DAR genes within the group.

Expression analysis of six BrDA1&DAR genes in various tissues such as root, stem, leaf, callus, flower, and silique suggested that most of the BrDA1&DAR genes are highly expressed in root, flower, silique, and callus while BrDA1.1, BrDA1.4, and BrDAR1.2 were found to be expressed strongly in all tissues in A. thaliana, AtDA1, AtDAR1, AtDAR2, and AtDAR4 were expressed highly in all tissues whereas AtDAR3 showed high expression in root and leaf, AtDAR5 and AtDAR6 highly expressed in root and stem and AtDAR7 showed maximum expression in flowers tissue (http://www.brassicadb.cn/#/Transcriptome/). B. rapa AtDAR1 expression is affected by ABA stress, while AtDAR4 is associated with cold stress [1,14]. In soybean (Glycine max), the GmaDA1 gene responded to drought, salt, ABA, alkali, and acid stress [15]. In this study, we found that BrDA1.1, BrDA1.4, BrDAR1.1, BrDAR2.3, BrDAR2.1, BrDAR2.2, BrDAR2.5, BrDAR6.1, BrDAR3.1 were mainly upregulated by ABA stress as compared to other hormones (Figure 8) and all these genes have abscisic acid-responsive elements in their promoter region (Figure 5). Under GA stress, BrDAR2.2, BrDAR6.1, and BrDAR6.3 were upregulated (Figure 8), while only BrDAR2.2 and BrDAR6.3 have GA-responsive elements in their promoter region (Figure 5). In response to SA, only BrDA1.2, BrDA1.3, BrDAR2.4, BrDAR6.2, and BrDAR6.3, expressed highly (Figure 8), and all genes have SA-related regulatory elements except BrDAR6.2, and BrDAR6.3 (Figure 5). While under IAA treatment, BrDA1.5, BrDAR1.2, BrDAR2.2, and BrDAR6.1 were upregulated (Figure 8), and only BrDAR1.2 and BrDAR6.1 contain IAA responsive elements in their promoter region (Figure 5). Interestingly, almost all members of the BrDA1&DAR gene family were upregulated by cold and heat stress (Figure 9). Still, in our cis-responsive element analysis, only BrDAR2.2, BrDAR6.2, and BrDAR6.3 have low temperature-related cis-element in their promoter region (Figure 5). While few members of the BrDA1&BrDAR gene family such as BrDA1.3, BrDA1.5, BrDAR1.1, BrDAR1.2, BrDAR2.2, BrDAR2.3, BrDAR6.2, and BrDAR6.3 were highly expressed under drought tolerance, out of these genes, promoter regions of three genes named BrDA1.5, BrDAR6.2, and BrDAR6.3 contain drought-responsive elements (Figure 5), and BrDA1.2, BrDAR1.3, BrDA1.5, BrDAR1.1, BrDAR2.2, BrDAR2.4, BrDAR6.2, and BrDAR6.3 were found to be upregulated by salinity stress (Figure 10a,b). These results indicate that the BrDA1&DAR gene family is involved in stress response.

Recently, many miRNAs have been identified and investigated in B. rapa that were involved in gene regulatory networks and different environmental stresses [16–18]. Present studies identified the four members of the br-miR164 family targeting one BrDAR6.1 gene, while br-miRN366 and br-miRN360 were found to target and regulate the two DA1 genes in B. rapa named BrDA1.4 and BrDA1.5 (Figure 6). Moreover, br-miR164 family members were involved in regulating heat tolerance in Chinese cabbage [19–21]. Our
results indicated that the expression of BrDAR6.1 got upregulated under heat stress, and the maximum expression was at 12 h. It would be interesting to investigate whether the br-miR164-BrDAR6.1 module functions in heat response.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13091577/s1, Figure S1: Protein structure analysis to highlight the presence of DA1-like domain in all family members of BrDA1& DARs. Table S1: The BrDA1&DAR family genes in A. thaliana, B. rapa, B. napus, and B. oleracea, Table S2: Details of gene duplication type and the ratio of Ka/Ks values of B. rapa, Table S3: The information of 8 identified motifs of BrDA1&DAR genes, Table S4: Details of cis-elements in the promoter regions of BrDA1&DARs, Table S5: Details of miRNAs directing BrDA1&DAR, Table S6: Primers used in this study for qRT-PCR analysis.

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