RESEARCH ARTICLE

Genome-wide identification and functional analysis of ARF transcription factors in Brassica juncea var. tumida

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Abstract

Auxin signalling is vital for plant growth and development, from embryogenesis to senescence. Recent studies have shown that auxin regulates biological processes by mediating gene expression through a family of functionally original DNA-binding auxin response factors, which exist in a large multi-gene family in plants. However, to date, no information has been available about characteristics of the ARF gene family in Brassica juncea var. tumida.

In this study, 65 B. juncea genes that encode ARF proteins were identified in the B. juncea whole-genome, classified into three phylogenetical groups and found to be widely and randomly distributed in the A-and B-genome. Highly conserved proteins were also found within each ortholog based on gene structure and conserved motifs, as well as clustering level.

Furthermore, promoter cis-element analysis of BjARFs demonstrated that these genes affect the levels of plant hormones, such as auxin, salicylic, gibberellin acid, MeJA, abscisic acid, and ethylene. Expression analysis showed that differentially expressed BjARF genes were detected during the seedling stage, tumor stem development and the flowering period of B. juncea. Interestingly, we found that BjARF2b_A, BjARF3b_A, BjARF6b_A, and BjARF17a_B were significantly expressed in tumor stem, and an exogenous auxin assay indicated that these genes were sensitive to auxin and IAA signaling. Moreover, eight of the nine BjARF10/16/17 genes and all of the BjARF6/8 genes were involved in post-transcriptional regulation, targeted by Bj-miR160 and Bj-miR167c, respectively. This analysis provides deeper insight of diversification for ARFs and will facilitate further dissection of ARF gene function in B. juncea.

Introduction

Auxin signaling is crucial for plant growth and development from embryogenesis to senescence. Many biological processes are associated with auxin signaling, including growth and development of root/stem, formation and differentiation of vascular tissue, apical dominance, and stress responsiveness [1, 2]. The effects of many active synthetic auxins on gene expression and regulation have been explored in crops, and the results have indicated that two types of
transcription factor families are required to regulate the expression of Auxin response genes: the ARF (Auxin response factor) family and the Aux/IAA repressor family [3]. ARFs act as transcription factors to control the expression of auxin- response genes by binding to auxin response element (AuxRE, TGTCTC motif) or its variations, such as TGTCCTC, TGTCAC, and TGTCGG, resulting in the repression or activation of these target genes in promoters of primary or early auxin-responsive genes. An ARF protein usually consists of three modular and portable domain: a DNA-binding domain, such as the B3-like DNA binding domain in the N-terminal (DBD); a middle region (MR), which functions as a repression or activation domain via a yeast-two-hybrid confirmatory assay; and a carboxyl-terminal dimerization domain (CTD), which can mediate homo- and hetero-dimerization of ARF or hetero-dimerization of ARF and Aux/IAA proteins [4, 5]. However, a minority of genes lack protein domains, due to each domain being functionally independent, such as ARF3, ARF13 and ARF17 in Arabidopsis.

To date, numerous studies have characterized the ARF gene family based on genomewide identification in many plant species, such as Arabidopsis [6], rice [7], maize [8], wheat [9], rapeseed [10], apple [11], barely [12], Vitis vinifera [13], tomato [14], Populus trichocarpa [15], Brassica rapa [16], and Brassica napus [17]. In Arabidopsis, 22 ARF genes (except one pseudogene) have been identified, which were categorized into three clades based on their amino acid sequence. Expression pattern analysis suggested that these genes are transcribed in a wide variety of tissues and plant development stages. However, 13 genes distributed on chromosome 1, which appear to be restricted to embryogenesis/seed development [18]. Additionally, combined with classical genetic approaches, the function of partial ARF family members’ has been confirmed. Of these TFs, ARF1 was the first identified TFs using a yeast-one-hybrid screen with a core motif, and has been found to respond to dark-induced senescence in leaves to regulate flower development [19, 20]. In ARF2 single mutant, leaf senescence, flowering, and floral abscission were delayed, indicating that its function is mediated by light [21]. ARF3 and ARF4 are involved in reproductive and vegetative tissue development [22]. ARF5 is associated with vascular strands and embryo axis formation [23, 24]. ARF7 participates in the response of impaired hypocotyls to blue light [25, 26]. ARF8 regulates fertilization and fruit development [27, 28]. In addition, T-DNA insertion mutants have shown that many ARF family members have overlapping functions in Arabidopsis [3, 29, 30]. In rice, 25 OsARF genes were identified using the AtARF protein sequence as a query for a BLAST search on the Oryza sativa genome [7]. Furthermore, nine OsARF proteins were predicted to function as activators and 16 as repressors. The transcription of OsARF1, OsARF5, OsARF14, OsARF21, and OsARF23 was affected with auxin treatment under light conditions, and OsARF1, OsARF2, OsARF16, OsARF21, and OsARF23 can be positively regulated by dark conditions [7]. An antisense phenotype revealed that OsARF1 is essential for vegetative and reproductive development associated with growth retardation, low vigor, and sterility phenotype [31]. OsARF8 regulates hypocotyl elongation and controls auxin homeostasis [7]. Furthermore, studies have shown that targeting of ARF10/16/17 by miR160 [32, 33] and ARF6/8 by miRNA167 [34, 35] is indispensable for various aspects of development, which might highly conserved in plants.

Tumorous stem mustard (Brassica juncea var. tumida Tsen et Lee), is one of the most important economic crops, and the raw material for Fuling mustard, in China. How to improve the yield (Tumor stem, the vegetative organ) of this crop is a key issue for the Chinese pickles industry. Auxin is vital for plant growth and development, exploring whether or not the effect of the auxin signaling pathway in crop production is meaningful. ARF gene family exists in tumorous stem mustard, but no evidence is available on the genome-wide identification and functional analysis of ARF genes in tumorous stem mustard, due to a lack...
of genome information. Genetic divergence between *Arabidopsis* and rice investigated by genome-wide analysis revealed that most *OsARFs* are related to *AtARFs*, and form sister pairs [3, 7]. The first assembly of *B. juncea* genome data has recently been published [36], making it possible and feasible to isolate functional gene families from *B. juncea* genome. Despite their crucial role in plant development, *ARF* family genes in *B. juncea* have not yet been identified and analyzed in detail. In this study, we identified *ARF* genes in *B. juncea* genome, determined their chromosomal distribution, gene and protein structure, and confirmed the expression of 65 *B. juncea* *ARF* genes by qRT-PCR. Furthermore, we analyzed cis-acting elements for *BjARF*-gene promoter regions and predicted potential targets for small RNA. Moreover, the phylogenetic relationship of *ARFs* between *Arabidopsis*, *B. napus*, and *B. juncea* were also compared, and partial specific *BjARF* gene expression was validated with an auxin treatment assay. We hope that this work will be helpful for more functional investigations of *ARFs* in tumorous stem mustard in future.

**Methods**

*B. juncea* *ARF* gene identification and chromosomal distribution

The *B. juncea* genome database was kindly supported by Zhejiang University. A local implementation of PlantTFDB V4.0 BLAST was used for sequence searching [37]. All known *ARFs* from *A. thaliana* (22 *ARF* genes, except a pseudogene) and *B. napus* (64 *ARF* genes) were used in initial protein queries. Gene names and IDs are listed in S1 Table, and *BjARF* genes were named/classified based on *A. thaliana* *ARF* genes. The hidden Markov model (HMM) profile (PF06507) of *ARF* family genes can be downloaded from PFAM (http://pfam.xfam.org/) [38], and the hmm build command from HMMER 3.0 software package can be used to transform it into a hidden Markov model sequential pattern. Then, we queried the “Yonganxiaoye” *B. juncea* genome database, with the following search parameters were followings: BLASTP, E value < 1e-10, identity > 70%, query coverage > 95%, and other parameters were defaulted. All non-redundant hits with expected values were collected and then compared with the *ARF* family in PlnTFDB [39] and PlantTFDB [37]. Subsequently, each candidate *ARF* gene was further confirmed base on the presence of the Auxin_res domain using SMART [40], CDD [41] and Inter-ProSca. The molecular weights and theoretical isoelectric points of the *BjARFs* were determined within Expasy [42]. Information on the physical location of the *ARFs* in *B. juncea* were collected from the corresponding GFF files, and mg2c was used to visualize the distribution of *ARF* genes on each *B. juncea* chromosome.

**Phylogenetic analysis of *BjARFs***

Phylogenetic analysis of multiple sequences was performed using full-length protein sequences of *ARFs* with ClustalW alignment. Then a phylogenetic tree was constructed with MEGA 7.0 software [43] by the Neighbor-Joining (NJ) method, carried out with 1,000 replicates bootstrap test.

**Gene structure and conserved motif of *BjARFs***

The exon/intron structures of the *BjARFs* were illustrated from alignments of genomic sequences and cDNA, and drawn with GSDS 2.0 [44]. Inputting the GFF files, MEME (suite 5.0.2) [45] was used to elucidate conserved motifs of *BjARFs*, and ran locally with the following parameters: maximum number of motifs -9, and width of motif constrained from 10 to 300. MAFFT Version 7 [46] was selected to present the Auxin_res domain.
**cis-acting elements analysis in promoter regions**

To investigate the putative cis-acting elements of 65 BjARFs candidate genes, BjARF promoter sequences (2000 bp upstream of the initiation codon ‘ATG’) were extracted from genome database of the *B. juncea*. PlantCARE [47] was used to obtain cis-acting elements.

**Gene expression profile analysis**

To analyze the expression profiles of BjARFs, we used a privately available RNA expression profile data, including leaf and stem of seedling stage (SS, 20-days after germination), mature stage (MS, 90-days after germination) and flower stage (FS, 150-days after germination) from three different cultivars, which named yonganxiaoye1 (YA1), yonganxiaoye2 (YA2) and yonganxiaoye3 (YA3). We used Log2 (FPKM) to calculate levels of BjARF expression, and their expression patterns were clustered by hierarchical clustering models and illustrated using a homemade R programming language.

**Prediction of BjARFs targeted by miRNAs**

BjARFs targeted by miRNA collected from the literature were predicted using psRNATarget [48], and the parameters were set as follows: maximum expectation of 3 and target accessibility (UPE) of 50.

**Auxin treatment and tissue preparations**

“Yonganxiaoye1” seeds were planted in field, and 10-days-old seedlings were transplanted into flower plots with nutritional soil substrate, and grown under natural conditions (16~24 °C with 16 h light/8 h dark). Root, leaf, tumor stem, and flowering bud tissues were collected at different growth stage. For the IAA treatment, the plant root was irrigated with 100 mL solution and plant surface was sprayed using an atomizer, two sets of seedling (20-days-old) and pre-stigmas (60-days-old) plants were irrigated/sprayed by water with and without 50 μM IAA, respectively. And then keep it for eight hours, tumor stem and leaf tissues were collected. All plant tissues were frozen in liquid nitrogen and subsequently stored at -80 °C until RNA isolation.

**Isolation of total RNA, reverse-transcription and real-time RT-PCR analysis**

RNA was isolated using the TRIzol method (Invitrogen, USA) and reverse transcribed to cDNA using M-MLV transcriptase (Roche, Switzerland). qRT-PCR was carried out using SsoAdvancedTM SYBR Green supermix (Bio-Rad, USA) on a Bio-Rad CFX96TM Real Time PCR System according to the manufacturer’s instructions. The relative quantitative method (2^ΔΔCT) [49] was used to calculate the fold change in the expression of target genes, and the *B. juncea* β-Actin gene was used as the internal control (S4 Table). Each measurement was made using two biological samples in triplicate per sample.

**Statistical analysis**

Statistical analysis was performed using paired-samples t-test (one-tail) by SPSS 19.0 software, the significant differences of gene relative expression levels was detected between the treatment and control.
Results

Genome-wide identification and chromosomal localization of ARF genes in B. juncea

To identify genes encoding ARF transcription factors in B. juncea, the Auxin-resp domain (a conserved region of auxin-responsive transcription factors, PF06507) was used to search against B. juncea proteins in HMMER software. All non-redundant hits with expected values were collected and then compared with the ARF family in PlnTFDB and PlantTFDB website. In addition, we verified the candidate ARF genes through the presence of the Auxin-resp domain using SMART, Prosca, and CDD. In total, 65 ARF genes were identified in B. juncea (S1 Table). Of those, we accurately named BjARF genes following their closest orthologs in A. thaliana and coded their different paralogs as a, b, c, etc., together with the order of the homologous chromosomes (S1 Table). The result shows that length of 65 ARF proteins varies from 157 to 1197 amino acids with predicted molecular weight of 17.85 to 132.65 kDa in B. juncea (S2 Table). In addition, analysis of chromosome location showed that those BjARF genes were distributed on all chromosomes present in the B. juncea genome (A1-A10, B01-B08), as well as seven contigs and three scaffolds comprised of Contig71, Contig94, Contig157, Contig533, Contig705, Contig1207, Contig2271, Super_scaffold_57, Super_scaffold_127 and Super_scaffold_187. We found that the numbers of BjARFs mapped on each chromosome were uneven and ranged from one to seven (Fig 1, S2 Table). In other species, segmental duplication, tandem duplication, and polyploidization were identified determined the genomic locations of the ARF gene family. We identified 26 pairs of segmentally duplicated ARF genes in the B. juncea genome (S1 Fig).

Phylogenetic relationships of ARF genes in B. juncea, A. thaliana, and B. napus

In total, 150 ARFs (including 65 obtained from this study in B. juncea, 22 from A. thaliana and 63 from B. napus) were used to construct a NJ phylogenetic tree by MEGA 7.0 software. As shown in Fig 2, we found that those ARF genes were clustered into three groups, named from I to III. In addition, the ARF orthologs were clearly distinguished in B. juncea. BjARF10, BjARF17 and BjARF16 were categorized into group I. BjARF4, BjARF3, BjARF6, BjARF8, BjARF5, BjARF7 and BjARF19 were categorized into group II. BjARF1, BjARF2, BjARF18, BjARF11 and BjARF9 were clustered in group III. However, ARF12, ARF13, ARF14, ARF15, ARF20, ARF21 and ARF22 were not identified in B. juncea in this study, AtARF12, AtARF13, AtARF14, AtARF15, AtARF20, AtARF21, and AtARF22 were categorized into a sub-group of Group III, and partial BjARFs and BnARFs were categorized into a novel sub-groups of Group III (Fig 2, S2 Fig), named ARFun1, ARFun2 and ARFun3, respectively.

Gene and protein structure of ARFs in B. juncea

To demonstrate structural diversity of BjARFs, the conserved motif and exon/intron structure from BjARFs promoters were analyzed. An unrooted NJ tree was constructed using 65 ARF protein sequences from B. juncea (Fig 3A). Their gene structures were analyzed by GSDS 2.0 and are displayed in Fig 3B. The results showed that Auxin_res domain was located in the N-terminal of all ARF genes, and the number of introns varies from one (BjARF17) to 17 (BjARF18b_O). Most genes within the same sub-family shared a similar structure and length, those in group I contained one to five introns less than those in group II and III, and members of group III had longer introns than members of group I and II, especially for BjARF11a_B, BjARF18b_O, BjARFun1a2_B, and BjARFun2a_A. Structural variation in genes provides...
valuable information on duplication events within gene families, these results suggest that different subfamily genes represent different functions in *B. juncea* development.

Furthermore, to better understand the sequence characteristics of *BjARF* genes, MEME was used to predict motifs based on 65 *BjARF* protein sequences, nine motifs named 1–9 were identified (Fig 4, S3 Fig). These conserved motifs contained between 21 (motif 8) and 60 (motif 1) amino acids. Each *BjARF* protein contain two (*BjARF10a_B*) to nine conserved motifs, with most containing nine motifs. Interestingly, most of the *BjARF* proteins contained motif 1 in the Auxin_resp domain. Moreover, we presented the Auxin_resp domain structures by constructing multiple alignments of all 65 *BjARF* proteins using MAFFT version 7 (S4 Fig). We concluded that similar gene structures and the motif architecture of *BjARF* orthologs were significantly clustered into the same phylogenetic tree, it suggesting they may have different roles in *B. juncea* development.

**Cis-acting elements prediction of *BjARF* gene promoter regions**

Cis-acting elements are vital sequences that are targeted by transcription factors to regulate gene expression. To investigate the putative functions of 65 *BjARF* genes in *B. juncea*, we obtained 2000 bp sequences upstream of the initiation codon ‘ATG’ and then used PlantCARE to predict *cis*-acting element. More than 234 types of putative *cis*-acting elements were identified in the promoter of *BjARFs* (S3 Table), including light responsive elements (G-box, TCT-
motif, GT1-motif, AE-box, Box 4, GATA-motif, I-box, TCCC-motif, MRE, GA-motif, 3-AF1 binding site, chs-CMA1a, LAMP-element, Box II, ATCT-motif, ATC-motif, GTGGC-motif, ACE, chs-CMA2a, Gap-box, GATT-motif, AT1-motif, CAG-motif, Sp1, chs-CMA2b, chs-Unit 1 m1, and L-box), promoter/enhancer elements (CAAT-box, TATA-box, CCAAT-box, A-box, AT-rich element, Box III, Unnamed__1, AT-rich sequence, 3-AF3 binding site, Box II-like sequence, MBSI, and HD-Zip 3), phytohormone response element [such as abscisic acid (ABRE), gibberellin (GARE-motif, P-box, TATC-box), MeJA (TGACG-motif), salicylic acid and auxin (TCA-element) and auxin (TGA-element, AuxRR-core, AuxRE)] (Fig 5), stress...
Fig 3. Phylogenetic tree of the ARF genes and genomic organization of ARF genes in *B. juncea*. A. NJ tree of BjARFs; the Auxin_resp domain was identified and the phylogenetic tree was constructed using MEGA 7, via the Neighbor-Joining method with 1000 bootstraps. Different groups of the ARF family are shown in different colors. B. Exon and intron structures of BjARFs, CDSs are shown in green, black lines connecting two CDSs represent introns.

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response elements (ARE, MBS, TC-rich repeats, LTR, WUN-motif and GC-motif), development and tissue specificity elements (CAT-box, O2-site, GCN4_motif, HD-Zip 1, RY-element, AACA_motif and a unnamed motif) and circadian control elements (circadian and MSA-like motif) (S3 Table). This suggests the most BjARF genes are involved in different biological processes, including responses to phytohormones, abiotic stresses and tissue development in B. juncea.

Prediction of small RNA for potential targets

To delineate the miRNA-mediated posttranscriptional regulation of BjARF genes, we searched the coding regions and 3' UTRs of all BjARFs for the targets of Bj-miR160 and Bj-miR167. The results showed that eight BjARF genes belonged to Group I that are complementary to the BjmiR160 mature sequences (Fig 6A). In addition, eight BjARF genes belonged to Group II that are complementary to the Bj-miR167c mature sequences (Fig 6B). The results suggest that BjmiR160 and BjmiR167c may specifically target these BjARF genes in B. juncea. BjmiR160 was located in the coding region of BjARF16a_A, BjARF16a_B, BjARF16b_A, BjARF16b_O,
BjARF17a_A, BjARF17a_B, BjARF17b_A, and BjARF10a_O (Fig 6A), and BjmiR167c was located in the coding region of BjARF6a_A, BjARF6a_B, BjARF6b_A, BjARF8a_B, BjARF8b_A, BjARF8c_A, BjARF8c_O, and BjARF8a_A (Fig 6B), respectively. These results revealed that post-transcriptional control of ARFs mediated by Bj-miR160 and Bj-miR167c are conserved in plants.

Fig 5. Analyze of cis-acting elements response to phytohormones in BjARFs promoters. Value of x-axis means the number of cis-acting elements which founded in BjARFs promoters.

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Fig 6. miR160/167c-mediated post-transcriptional regulation of BjARFs. (A–H). Prediction of the BjARFs regulated by Bj-miR160; (I–P). Prediction of the BjARFs regulated by Bj-miR167c. miRNA target sites (Black) with the nucleotide positions of BjARF transcripts are shown, and the Auxin_res domain (Blue) with the nucleotide positions of BjARF transcripts are shown. The RNA sequences of each complementary site from 5' to 3' and the predicted miRNA sequence from 3' to 5' are indicated in the expanded regions.

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Expression profiles of BjARF genes in B. juncea

A certain levels of gene expression is vital for determining gene function. Therefore, we analyzed the expression profiles of all identified 65 BjARFs available from our private RNA expression profile data. A heat map showing the expression of BjARFs was constructed using homemade R and is shown in Fig 7A. BjARF genes were divided into four groups based on their expression profiles: BjARF10a_O, BjARF1a_A, and BjARF7a_A were highly expressed in all tissues during the leaf of seedling stage (SS), leaf and stem of flowing stage (FS), leaf and stem of mature stage (MS) from YA1 (yonganxiaoye1), YA2 (yonganxiaoye2), and YA3 (yonganxiaoye3) respectively. Interestingly, BjARF10a_O was highly accumulated in the stem of flowing period (FS) and mature stage (MS), and BjARF1a_A was preferentially expressed in leaf of FS and MS. In addition, BjARF2b_A and BjARF6b_A were found specifically expressed in stem from YA2 and YA1 of FS and MS, respectively. Further, BjARF2b_A, BjARF2a_B and BjARF16b_O were specifically expressed in leaves at different periods of B. juncea development. However, no other BjARF genes were significantly detected in all tested tissues (Fig 7A).

These candidate BjARF genes, which may be significantly expressed in different tissues, were validated using qRT-PCR. Plant seedlings (20-days after germination), plant leaves in the vegetal period, tumor stem of vegetal period (60-days after germination, VP), and flower period (150-days after germination, FP), flower and legume of Yonganxiaoye 1 tissue were collected for RNA isolation. Analysis of expression patterns showed that, BjARF16b_O and BjARF2b_A were significantly expressed during early plant growth in leaves (Fig 7E and 7F),

**Fig 7. Expression pattern of BjARFs.** A. Expression profiles of BjARFs from RNA-seq in different tissues and cultivars. Color represents BjARF expression levels: Log2 (FPKM). The phylogenetic relationship is shown on the left. RNA expression data were selected as follows: leaf (L) of the seedling stage (SS), leaf and stem (S) of the flowing period (FP), leaf and stem of the mature period (MP) from YA1 (yonganxiaoye1), YA2 (yonganxiaoye2) and YA3 (yonganxiaoye3), respectively. B–M. Validation of candidate BjARFs using qRT-PCR, based on altered expression levels. From left to right representing seedling, leaf (VP), tumor stem (VP), tumor stem (FP), flower, and legume. Error bars show the standard error calculated from three biological replicates. Values are presented as the mean ± SEM.

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BjARF3b_A and BjARF6b_A were highly expressed in the tumor stem (Fig 7C and 7D), and BjARF17a_A, BjARF2a_B, and BjARF16b_A were significantly expressed during the reproductive stage of B. juncea (Fig 7G–7I). Only BjARF17a_B was exclusively expressed in the stem nodule during B. juncea stem tumor development (Fig 7B). However, expression of BjARF1a_A, BjARF7a_A, BjARF10a_O, and BjARF19a_A was widespread (Fig 7J–7M).

**Induction of BjARF gene expression by exogenous auxin**

To confirm whether candidate genes are induced by auxin signal, 20-days-old seedlings and 60-days-old plants were treated with and without 50 μM IAA for 8 h. The seedlings, plant leaf, and tumor stem were collected and relative gene expression of eight candidate BjARF genes significantly expressed in different plant tissues was determined. The results showed that the expression of most BjARF genes was induced in specific organization and development period of plant. For example, expression of BjARF2b_A, BjARF2a_B, BjARF16b_A, and BjARF16b_O were significantly increased in leaf exposed to 50 μM IAA (Fig 8A, 8E, 8F and 8H); expression of BjARF3b_A, BjARF6b_A and BjARF17a_B were significantly increased in tumor stem under exogenous auxin treatment (Fig 8B–8D). In contrast, expression of BjARF17a_A was induced following with treatment with exogenous auxin in seedlings (Fig 8G). These results suggest that BjARF genes are sensitive to auxin, and that BjARFs play crucial roles during B. juncea development and are regulated by endogenous plant hormones.

![Fold induction of BjARF genes in response to exogenous auxin](https://doi.org/10.1371/journal.pone.0232039.g008)
Discussion

The theory of U’s triangle illustrates the evolutionary relationship of the cruciferous plants *B. nigra*, *B. oleracea*, and *B. rapa*. U’s triangle showed that *B. juncea* originated from *B. rapa* and *B. nigra* through hybridization and genome duplication [50]. *B. juncea var. tumida* is a popular vegetable in China, and becoming geographical indication products of Chongqing city. In recent years, ARF genes have gained widespread attention due to the large number of family members and their response to most of life processes in plants, and ARFs were genome-wide identified in Arabidopsis, rice, maize, and wheat successively [6, 7, 8, 9]. In this study, we characterized 65 *B. juncea* ARF genes by genome-wide analysis (S1 Table), which is a higher number than reported in other species. We suspect that this may be due to plant evolution and by *B. juncea* belonging to an allotetraploid. In recent years, researches have demonstrated that segmental duplications were existed and play vital roles in the gene expansion of ARF transcription factor gene family in many plants [16, 17]. Here, 26 pairs of segmentally duplicated ARF genes were identified in the *B. juncea* genome. Phylogenetic analysis revealed that each ARF genes was conserved in Cruciferae [6, 16, 17], especially between *B. juncea* and *B. napa*. We named the BjARFs according to the homologous ARF gene in *Arabidopsis* (S1 Table). Interestingly, we found that AtARF12–15 and AtARF20–22 were categorized into a sub-group of Group III, and 11 BjARFs and 14 BnARFs were categorized into another sub-group of Group III. Thus, we speculate that *B. juncea* evolved a set of unique ARF genes to adapt its development compared to Arabidopsis, and that *B. napa* was more closely related to *B. juncea* in evolution. It suggested that gene function of unique genes are more similar between *B. juncea* and *B. napa*, we should give priority to *B. napa* as a reference for gene function research in future. These unique genes may present functional specificity in different plant species, explaining the existence of a larger family in *B. juncea*. In addition, gene structures and conserved protein motifs in BjARFs were found in different ARF orthologs with a diverse number of introns, we speculated that different ARF orthologs shared similar functions.

Analysis of BjARF gene expression showed that these genes could be divided into four groups, and partial BjARF members were significantly expressed in plant tissues or different varieties. Many ARFs (AtARF2, AtARF7, AtARF19, OsARF1, OsARF5, OsARF19, OsARF24, SIARF2, and SIARF19) were found to be involved in regulating the development of plant roots, stems, leaves and flowers [9, 20, 31, 51, 52]. In this study, 12 candidate genes were subjected to expression pattern analysis. Interestingly, we obtained two BjARF genes that were significantly expressed in plant seedlings and leaves during early plant development, three BjARF genes significantly expressed in plant flowers and legumes that may be associated with plant reproductive and embryonic development, four BjARF genes that were specifically expressed in stem during stem development. In addition, four BjARF genes were widely expressed in the whole plant during development process. Therefore, it is speculated that BjARF members with the same expression patterns share similar biological functions. For example, BjARF17a_B, BjARF3b_A, BjARF6b_A, and BjARF16b_O may play key roles in stem tumor intumescence.

In plants, miRNAs play a regulatory role in plant cell by negatively affecting gene expression at the post-transcriptional level. A number of pairs of microRNAs and their respective mRNA targets were predicted by database analyses [53]. Among these, miR160 was shown to target ARF10/16/17 and miR160 was shown to target ARF6/8. We proposed that miRNA160/167 might also response to auxin signaling via the negative regulation of auxin response factors in *B. juncea*, and we demonstrated that 16 BjARF members were putative targeted by miR160/167. In *Arabidopsis*, transgenic expression of miR160-resistant of ARF10, ARF16 and ARF17 resulted in pleiotropic phenotypes associated with their over-accumulation [54, 55].
results suggest that miR160 is essential for seed germination, root-cap formation and in vitro shoot regeneration. In soybean, miR160 was confirmed as a link between auxin and cytokinin action, by balancing auxin activity, through regulation of target repressor ARFs, for proper nodule development [56]. In tomato, miR160 was found to be regulate auxin-mediated ovary patterning as well as floral organ abscission and lateral organ lamina outgrowth [57]. Previous studies have showed that miR167 target auxin response element (AuxRE, TGTCCT motif or other similar sequences) and response to auxin signaling in early auxin-inducible gene promoters [58], and current study confirmed that NtMIR167a-NtARF6/8 is critical for plants in regulating Pi starvation tolerance [59]. We believe that the mechanism of miRNA160/167 involved in Auxin-mediated regulates mustard development will be revealed in our near future study.

Furthermore, we confirmed that eight significantly expressed candidate genes respond to exogenous hormones. When plants were exposed to IAA, expression of most of these BjARF genes was induced during a specific organization or development period of the plant (Fig 8). The relative expression of AtARF4, AtARF16, AtARF19, OsARF1, and OsARF23 increased in response to auxin [8, 21–24, 31], while that of AtARF5, AtARF14, and AtARF15 decreased [23, 24]. In this study, most of the BjARFs were induced by exogenous auxin and auxin responsive elements were detected in promoter regions (S3 Table). In addition, the expression of BjARFs may also be regulated by miRNA, AtARF6 and AtARF8 were reported to be targets of miR167 during the regulation anther and ovule development [53, 60–62], while AtARF10 and AtARF16 were reported to be targets of miR160 during root cap formation [29, 53]. In present study, BjARF10, BjARF16, and BjARF17 were predicted to be target genes of Bj-miR160 (Fig 6A), while BjARF6 and BjARF8 were predicted to be target genes of Bj-miR167c (Fig 6B), suggesting that Bj-miR160 and Bj-miR167c may participate in regulation of B. juncea growth and development by responding to changes in ARF gene expression. Therefore, the mechanism underlying the auxin-mediated regulation of BjARFs needs to be clarified, and regulation of the expression of other BjARF genes should be further explored.

Accumulating evidence suggests that auxin signaling is mediated by ARFs, is usually involved in plant morphogenesis, such as apical dominance, tropic responses meristem elongation, root development, and shoot elongation, as well as responses to various abiotic stresses [1, 2]. AtARF1 and AtARF2 were confirmed to control leaf senescence, silique ripening, and floral organ abscission independently of the ethylene and cytokinin response pathways in Arabidopsis [20]. In this study, BjARF2b_A and BjARF16b_O was significantly expressed during early plant growth in leaf, BjARF2b_A, BjARF2a_B, BjARF16b_A, and BjARF16b_O expression were conducted by exogenous auxin in leaves. Additionally, a recent study indicated that AtARF3 integrates the functions of AG and AP2 in floral meristem determinacy, while AtARF4 is associated with organ polarity. ARF5 is required for embryonic roots and flowers [22–24] and ARF8 regulates fertilization and fruit development. ARF2, ARF4, ARF5, and ARF17 was confirmed be associated with anther development, pollen formation and gametophyte development [63, 64]. ARF18 affects the yield of rapeseed through regulates seed weight and silique length [10]. At present study, BjARF17a_A, BjARF2a_B, and BjARF16b_A may response to regulate inflorescence development and reproductive in tumorous stem mustard, which is expressed extremely high during reproductive stage of B. juncea (Fig 7G–7I). Furthermore, we have obtained three candidate genes may associate with tumor stem development, it may provide new insight for yield improvement of tumorous stem mustard (Fig 7B–7D). In summary, 65 BjARFs were identified in B. juncea, and together with phylogenetic analysis, GSDS analysis, expression pattern analysis, and exogenous IAA treatment, the functions of partial BjARF gene members were predicted. Notably, some genes may regulate tumor intumescence, which is crucial for B. juncea production. The potential functions of other family members need to be evaluated and our speculations should be further explored. This would be helpful for future
research to focus on the functional analysis of tissue and development-dependent ARF transcription factors.

Conclusions
In this study, a total of 65 ARF genes were identified and distributed on all chromosomes present in the B. juncea genome. All the family members have similar gene structures and protein domains to the auxin response factors of Arabidopsis. Several phytohormone response elements were found in the promoter region of BjARFs, indicating these genes may regulate plant development, which could benefit from a more targeted follow-up study. Bj-miR160 and Bj-miR167c were predicted to regulate BjARFs gene expression through post-transcriptional, which is conserved in plants. Gene expression analysis showed that some BjARF family genes exhibit a special expression pattern inducted by exogenous auxin, and we speculated that some family members may be involved in the regulation of tumorous stem mustard yield. Together, this work will be helpful for more functional investigations of ARFs in tumorous stem mustard in future.

Supporting information
S1 Table. Gene name and ID of ARFs in B. juncea, Arabidopsis and B. napus. (XLSX)
S2 Table. Characterization of ARF family genes in B. juncea. (XLSX)
S3 Table. Cis-acting elements of BjARF genes in promoter regions. (XLSX)
S4 Table. List of primers for qRT-PCR analysis of candidate BjARFs. (XLSX)
S5 Table. Expression patterns of global expression gene in various tissues of Brassica juncea var. tumida. Including leaf of Seedling stage (SS), leaf and stem of flowering stage (FS), leaf and stem of mature stage (MS) from YA1 (yonganxiaoye1), YA2 (yonganxiaoye2) and YA3 (yonganxiaoye3) respectively. (XLSX)
S1 Fig. Phylogenetic tree of the ARF gene family in B. juncea annotated with collinear and tandem relationships. Curves connecting pairs of gene names suggest the collinear relationship. This annotated tree is output from ‘family tree plotter’ of MCscanX software. (PNG)
S2 Fig. Phylogenetic relationship of special ARF genes from B. juncea, A. thaliana, and B. napo. The phylogenetic tree was constructed using MEGA 7.0, the maximum likelihood method with 1,000 bootstraps. The different special of the ARF family are represented in different colors. (PNG)
S3 Fig. Motif Logos of 9 conserved motifs of BjARF proteins. (PNG)
S4 Fig. Alignment of the Auxin_resp domain in BjARF proteins. Multiple sequence alignment was performed using MAFFFT version 7. (PNG)
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