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Genome wide identification and expression profiling of Bcl2 associated anthanogene family cochaperones reveals abiotic stress response in *Ricinus communis* L.

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Castor (*Ricinus communis* L.), a member of the Euphorbiaceae family, is a non-edible oilseed crop extensively cultivated in arid and semi-arid regions worldwide for its diverse industrial uses. The B-cell lymphoma 2 (Bcl-2)-associated athanogene (*BAG*) family is a diverse and well-conserved co-chaperone family present in both plants and mammals. BAG proteins interact with a wide range of proteins, regulating various functions, including stress response, growth, and development. However, the function of *BAGs* in oilseed crops like castor remains largely unknown. In this study, we discovered 9 BAG protein family members (*RcBAGs*) in castor through genome-wide scanning. We investigated chromosomal localization, performed in silico promoter analysis, conducted phylogenetic and synteny analyses, and examined gene architecture. Additionally, we predicted protein–protein interactions and assessed the responses of these genes to various abiotic stresses and hormones. Based on their cellular localization, the *RcBAG* family was categorized into nuclear, chloroplastic, and cytoplasmic groups. Syntenic gene pairs across different crops also validated the importance and functional conservation of these *BAG* genes during evolution. Furthermore, in *Ricinus communis*, the *RcBAG* genes were scattered unevenly throughout seven of the 10 chromosomes. The study reveals that *RcBAG* genes are crucial for stress management and castor growth, responding to abiotic stimuli through distinct regulatory pathways. Quantitative real-time polymerase chain reaction (qRT-PCR) investigation revealed that 9 distinct *RcBAG* genes were strongly induced after cold and heat treatments. Functional analysis and protein–protein interactions were used to predict the potential regulatory network of RcBAGs, revealing tight networking and signaling with HSP proteins. This study provides a foundation for future research into the molecular mechanisms and regulatory processes during *R. communis* growth, development, response to various stressors, and protein interactions.

Keywords *Ricinus communis*, Non-edible oilseed crop, BAG protein family, Co-chaperones, Abiotic stress, Heat shock proteins

Abbreviations

BAG	B-cell lymphoma 2 (Bcl-2)-associated athanogene
PCD	Programmed cell death
MDA	Malondialdehyde
H_2O_2	Hydrogen peroxide

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HT	High temperature
qRT-PCR	Quantative real time polymerase chain reaction
MEME	Multiple Em for motif elicitation
MeJA	Methyl Jasmonate
PPI	Protein–Protein Interaction
GUS	β-Glucuronidase
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
UPR	Unfolded protein response

The *Euphorbiaceae* family includes approximately 8,000 species that are distributed across a wide range of climates globally [1,2]. Castor (*R. communis* L.), a prominent specie of this family, is extensively cultivated in dry, tropical, and arid regions around the world [3]. Castor is widely cultivated in many countries due to its great level of environmental adaptability [4,5]. Major producers of castor seeds include India, Mozambique, China, and Brazil, which account for 85.81%, 6.11%, 1.93%, and 1.01% of global production, respectively. In 2018, 1.30 million hectares of land produced 1.40 million tons of castor seeds, with an average yield of 1076.5 kg per hectare [6,7]. In India, Gujarat is known as the "castor bowl of India," producing over 70% of the country's castor, thus making it the leading state in castor production [8].

Castor beans, a major crop with several industrial applications, its high ricinoleic acid concentration and other distinctive features. Castor seed oil is different from other vegetable oils in that it contains up to 90% ricinoleic acid, a triacylglycerol composed of glycerol and fatty acids [9]. This high concentration has a substantial economic impact, benefiting the toxicological, pharmaceutical, and cosmetic industries [10–12]. Furthermore, castor plants exhibit remarkable resistance to a wide range of abiotic stresses, enhancing their viability in challenging environments [13,14]. This resilience, coupled with its economic potential, makes castor a valuable crop for developing countries, particularly for small-scale farmers in dry locations [15,16]. Abiotic stress is widely recognized as a major environmental factor that threatens high yield and large-scale agricultural production [16,17]. Plants have the potential to adjust their morph-physiological, biochemical, and molecular pathways in response to the harsh environmental conditions imposed by abiotic stressors [18,19]. Castor bean (*Ricinus communis* L.), though native to tropical regions, is now widely cultivated in subtropical and arid environments due to its adaptability and industrial value. Castor is highly sensitive to extreme temperatures, with cold stress during seed imbibition limiting seedling emergence and growth [20], and heat stress during early development further affecting its establishment [21]. Recent transcriptomic studies have identified over 2400 differentially expressed genes under cold stress conditions (20 °C), with a notable upregulation of phenylpropanoid-related genes, suggesting their involvement in chilling-responsive germination regulation [20]. In response to heat stress, lipidomic and transcriptomic analyses revealed that castor bean undergoes substantial lipid remodeling, especially the accumulation of polyunsaturated triacylglycerols (TAGs), which may serve as intermediates in lipid turnover and stress adaptation [22]. Additionally, stress-associated proteins (SAPs) in castor have shown distinct tissue-specific and stress-induced expression patterns, highlighting their potential role in coordinating abiotic stress responses through pathways that may function independently of classical hormonal signals [23].

The BAG family is a diverse and evolutionarily conserved group of co-chaperone proteins found in both plants and animals [24]. These proteins are essential for controlling development, growth, and stress responses because they interact with a wide range of protein targets [25]. In plants, BAG proteins have been shown to be involved in numerous physiological processes, including response to abiotic stressors such as drought, salt, and temperature extremes [26,27]. Recent studies for genome-wide identification have significantly enhanced our knowledge of the *BAG* gene family across different plant species. Plant BAG proteins, for example, have been discovered in *Arabidopsis* and are being studied for their capacity to multitask across a range of cellular signal transduction pathways, as well as their role in plant development and stress [28,29]. They act as co-chaperones, and a cells BAG protein to HSP70 ratio is critical for its correct functioning. BAG protein management is critical for cell survival in stressful conditions because higher BAG protein-to-HSP70 ratios reduce HSP70s refolding activity [30]. Plant functions such as development, stability, stress response, and programmed cell death (PCD) are mediated by BAG proteins. It has been recently shown that *AtBAG2* functions as the only molecular chaperone in *Arabidopsis* [31]. Similarly, *Arabidopsis BAG2* and *BAG6* showed response to numerous abiotic stresses [26]. AtBAG4-7 is required for several *Arabidopsis* BAG proteins, including abiotic stress-induced cell dying, production of ROS, senescent of leaves, autophagy, and heat and cold tolerance [32]. In contrast, the *atbag4* mutant reacts well to salt treatment. Overexpression of *OsBAG4* improves broad-spectrum disease resistance in rice, revealing that *OsBAG4* is an important driver of disease resistance [33]. *AtBAG5* regulates senescence of leaves by acting as a connection between the calcium pathway and the Hsc70 chaperone [30]. 11 *BAG* genes were discovered in tomato, a model crop, and were shown to be substantially conserved between plants [34]. The stress hormones abscisic acid (ABA) and ethylene affect their expression [34]. Overexpression of *SlBAG2* and *SlBAG5b* may protect tomato leaves from dark stress and delay senescence [35]. Throughout plant development, *SlBAGs* exhibit a variety of particular tissue-specific expression patterns, most notably during fruit growth and maturation [36]. *SlBAG9* overexpression has recently been demonstrated to make tomato plants more susceptible to high temperatures, resulting in lower chlorophyll content and a lower net photosynthetic rate [37]. The increased ion leakage, malondialdehyde (MDA) content, and hydrogen peroxide (H_2O_2) content suggested that *SlBAG9* overexpression enhanced the degree of high temperature (HT) induced membrane oxidation [38]. SlBAGs in tomatoes regulate heat stress and PCD, interacting with Hsp70 protein and Hsp20s [39,40]. Knockout mutants of *MAPK2* and *BAG2* genes show decreased activity of caspase 3 and caspase 9, a key enzymes involved in PCD [39]. BAG8 interacts with PP2A, which regulates stomatal growth, and Hsp70, which modulates photosynthesis, to improve photosystems and antioxidant systems and boost tomato cold tolerance [37]. However, HSP70 is involved

in BAG9-mediated thermotolerance in tomatoes by ensuring photosystem stability and increasing the efficiency of the antioxidant system [42]

Apart from Arabidopsis [27,43] and tomato [34], BAG proteins have been discovered in rice [36,44,45], maize [46], moss [47] banana [48], tobacco [49], chickpea [50] and soybean [51]; however, understanding of BAG proteins in other plants species, notably fruit crops and legume plants, is limited. Castor (*R. communis* L.) is an oilseed crop in the *Euphorbiaceae* family with a high concentration of the unique fatty acid ricinoleic acid, making it a valuable source of castor oil, which is used to make high-quality lubricants [52]. So, in this study, we for the first time have identified *BAGs* genes in *R. communis* L. and carried out genome-wide identification like gene structure, motif analysis, chromosomal locations, conserved domains analysis, Synteny analysis, and their expression during developmental stages and cold and heat stress. Furthermore, the evolutionary relationships between several model and legume plant species and castor bean *BAG* genes (*RcBAGs*) were investigated. *RcBAG* promoter regions are also analyzed in silico to identify cis-acting regulatory factors. The potential regulatory network of RcBAGs was anticipated using functional analysis and protein–protein interactions, which demonstrated tight networking and signaling with HSP proteins. *RcBAG* expression patterns were studied in different tissues, and stress (cold and heat stress) treatments. Furthermore, quantative real time polymerase chain reaction (qRT-PCR) of different *RcBAGs* genes revealed that *RcBAG1*, *RcBAG2*, *RcBAG5*, and *RcBAG6* are expressed in leaves, stem, flower and roots, while *RcBAG3*, *RcBAG4*, *RcBAG7*, *RcBAG8*, and *RcBAG9* genes are expressed in stem, flower, and leaves. *RcBAGs* expressions are regulated by cold and heat stress at different time points. The findings of this study may serve as a foundation for future castor bean research. This particular study can assist elucidate the role of *BAGs* in castor and pave the way for future studies on gene function.

Materials and methods

Identification of *BAG* genes in *R. communis* L. and other eudicot genomes

Protein sequences for the found genes were acquired using the model organism Arabidopsis thaliana. AtBAG6 protein sequence was downloaded from Phytozome 13 (https://phytozome-next.jgi.doe.gov/) (**Table S2**) and used as query sequence to blast against the *R. communis* L., *Arabidopsis thaliana*, *Cicer arietinum*, *Linum usitatissimum*, *Lupinus angustifolius, Trifolium pretense, Medicago truncatula, Glycine max, Phaseolus vulgaris, Vigna radiate, Vigna unguiculata, Lotus japonicas, Manihot esculenta, Lupinus albus, Populus trichocarp* and *Vitis vinifera,* genomes to fetch out all the family genes. Protein sequences obtained from phytozome v13 were utilized to do Markov Model (HMM) profiling of the BAG domain (PF02179) in order to identify BAG domain-containing proteins in all BLASTP results (**Table S2**). Furthermore, the SMART and Pfam databases were examined to ensure that the identified proteins included the BAG domain (**Table S2**). Specifically, a BLAST E-value cutoff of < 1e−10 and a minimum sequence identity of 75% were applied to ensure the inclusion of only high-confidence alignments. All of the redundant BAG protein sequences were removed, and Coding, genomics and protein sequences of all BAG genes were downloaded.

Phylogenetic analysis of *BAG* family in *R. communis* L.

Multiple sequence alignment of (protein sequences of BAG) from R. communis, Arabidopsis thaliana, Cicer arietinum, Linum usitatissimum, Lupinus angustifolius, Trifolium pretense, Medicago truncatula, Glycine max, Phaseolus vulgaris, Vigna radiate, Vigna unguiculata, Lotus japonicas, Manihot esculenta, Lupinus albus, Populus trichocarp and Vitinus vinifera genomes, were conducted by using MEGA 11 software [53]. Bootstrap value with 1000 replicates was used for the reliability of groups.

Gene structure analysis of *BAG* family in *R. communis* L.

Genomic, protein and full length CDS sequences of all *BAG* genes of *R. communis* L. were downloaded from the Phytozome 13 for gene structure. Gene Structure Display Server (GSDS) tool (http://gsds.cbi.pku.edu.cn/) was used to present the number of introns, exons, upstream and downstream regions of all *BAG* genes [54]. The chromosomal locations for *RcBAGs* were found using the NCBI database (http://www.ncbi.nlm.nih.gov/).

Motifs display of BAG family proteins in *R. communis* L.

Number of motifs of BAG proteins sequences of *R. communis* L. was determined using the Multiple Em for motif Elicitation (MEME) software (http://meme.nbcr.net/ meme4_1/cgi-bin/meme.cgi). The software settings were configured to identify a maximum of ten motifs, with other default values [55].

Domain assessment of *BAG* members in *R. communis* L.

For domain analysis of all BAG proteins sequences of *R. communis*, protein sequences were subjected to CDD NCBI software (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and hitdata files were downloaded. Then, for domains visualization hit data files were subjected to TB Tool software [56,57].

Synteny analysis of *RcBAG* genes

Genomic and GFF3 files of five organisms (*R. communis, Solanum lycopersicum, Populus trichocarpa, Manihot esculenta, and Arabidopsis thaliana*) were downloaded from the (Phytozome.13) database [58]. These organisms belong to the same family, order, or class as *R. communis* and were picked to understand the evolutionary relationship and functional conservation among these plants. All of the genomic sequences and GFF3 files were used in MCscanX to generate subsequent files. Different gene pairs of 9 *BAG* of interest were identified in all four chosen organisms. Advanced circus of TBtools v. 2.106 was used to visualize these results [59]

In silico promoter analysis of *BAG* family members in *R. communis* L.

The 1 kb upstream sequences from the start codon of all *BAG* genes of *R. communis* L. were retrieved from the Phytozome 13 (https://phytozome-next.jgi.doe.gov/blast-search). These promoter sequences were analyzed by using (plantCARE) database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for the identification of cis-acting elements. Data of cis-acting elements obtained from plantCARE database were subjected to TB Tool for Heatmap to present all promoters ratio present in all BAG proteins sequences of *R. communis* L.

R. communis L. BAG proteins physicochemical properties and subcellular localization

Physiochemical properties of all BAG proteins sequences of R. communis were identifies using Expasy Protparam tool (https://web.expasy.org/protparam/) and sub-cellular localization were identified using WoLF PSORT and CELLO Prediction tools (https://www.genscript.com/wolf-psort.html) (Table S2).

Plant materials and expression profiling studies by qRT-PCR

The Castor bean (32,473 cultivar) seeds used in experiments were collected from National Genebank, Plant Genetic Resources Institute, National agriculture Research Council, Islamabad, Pakistan. The seeds were soaked for 24 h in water, and then shifted to pots (soil-sand mix in a 3:1 ratio) at 22 °C in greenhouse 16 h/8 h light/dark cycle [5,60]. Tissue samples from flowers, stems, seeds, roots, old leaves, and young leaves were collected at different stages of development and utilized to tissue specific expression of *RcBAGs*. The tissues were instantly frozen in liquid nitrogen and stored at -80 °C until further use. Total (RNA) extraction, cDNA preparation, and (qRT-PCR) (primers listed in **Table S1**) were done on 21-day-old caster seedlings under stress and control conditions to confirm the expression of the RcBAG gene for qRT-PCR investigation of cold and heat stressors [34,48,61,62].

Results

Genome-wide analysis, gene structure, chromosomal locations and domain examination of *RcBAGs*

Protein sequences obtained from Phytozome v13 and Ensemble Plant were utilized to conduct Markov Model (HMM) profiling of the (BAG) domain (PF02179) in order to identify BAG domain-containing proteins among all BLASTP resultant proteins. The SMART and Pfam databases were utilized to ensure that the found proteins have the BAG domain. The coding, genome, and protein sequences for each *BAG* gene were obtained, and any duplicate BAG protein sequences were removed. A total of 9 proteins in *R. communis* have been identified, and their nomenclature has been assigned based on *Arabidopsis* homologs (Table 1). When the WoLF PSORT program was used to validate the anticipated targeting signaling peptides for RcBAGs proteins, most proteins were found to be located to the nucleus, chloroplasts, and cytoplasm (Table 1). Studied RcBAGs and other taxa investigated, reflect ancient eukaryotic BAG-domain proteins as demonstrated in Fig. 1A. Conserved pattern of introns and exons distribution including their up-stream and downstream regions was determined in all *BAG* genes of *R. communis* L. *RcBAG6* gene has only exonic region without intron and upstream regions while remaining genes have intron, exon and upstream/downstream regions. *RcBAG1* have 2 exons and 1 intron, *RcBAG3*, *RcBAG7*, *RcBAG8* and *RcBAG9* have 4 exons and 3 intron regions (Fig. 1B). Similarly, *RcBAG2* have 2 exons, 1 intron and one upstream/downstream while *RcBAG4* has two upstream/downstream regions and one intronic region respectively (Fig. 1B). The findings also indicate that all RcBAG proteins include ten common motifs and one highly conserved motif linked with the BAG domain (Fig. 1C).

One of the most significant aspects of genomic research is determining the location of the gene in order to conduct a more thorough examination and understanding of its function. It provides a framework for identifying the amino acid that regulates the stress or characteristic by analyzing sequence alterations in the gene family. On the basis of this, the chromosomal distribution of the nine genes in the *RcBAGs* family was examined using the TBtools program. The *RcBAG* genes are irregularly distributed on 10 chromosomes, including only one gene is found on chr1, chr2, chr4, chr6, chr7 and chr10, while three genes are found on chr5 respectively (Fig. 1D). To better understand the domain architectural features of (RcBAG proteins), domain analysis was performed using MEME software (**Table S2**). According to results, in all the identified RcBAGs, BAG domain is highly conserved in them. RcBAG7, RcBAG8 and RcBAG9 have the ubiquitin like superfamily domain in addition to the BAG

Gene Name	Transcript ID	Gene identified	Sub-cellular localization	Protein Length	MW (kDa)	PI	Negatively charged residues	Positively charged residues	Aliphatic index	GRAVY
RcBAG1	30174.m008685	30174.t000082	Nuclear	1170	131,576	5.01	205	147	66.91	−0.97
RcBAG2	29630.m000792	29630.t000017	Chloroplast	465	52,828.9	7.36	59	59	73.74	−0.78
RcBAG3	30147.m014306	30147.t000577	Chloroplast	353	38,976.3	9.59	39	49	75.64	−0.669
RcBAG4	29609.m000597	29609.t000024	Nuclear	182	20,507.1	5.26	28	22	84.62	−0.433
RcBAG5	29601.m000432	29601.t000004	Chloroplast	152	16,992.1	4.95	30	23	79.54	−0.661
RcBAG6	29929.m004607	29929.t000110	Nuclear	389	44,086.5	5.23	71	58	67.35	−0.827
RcBAG7	30140.m000571	30140.t000006	Chloroplast	265	29,489.9	5.77	43	39	92.6	−0.346
RcBAG8	30190.m011124	30190.t000360	cyto_nucl	301	34,421.9	9.56	39	52	92.56	−0.645
RcBAG9	30059.m000462	30059.t000008	Cytoplasmic	296	33,287.8	8.96	34	39	86.59	−0.342

Table 1. The List of *RcBAG* family genes.

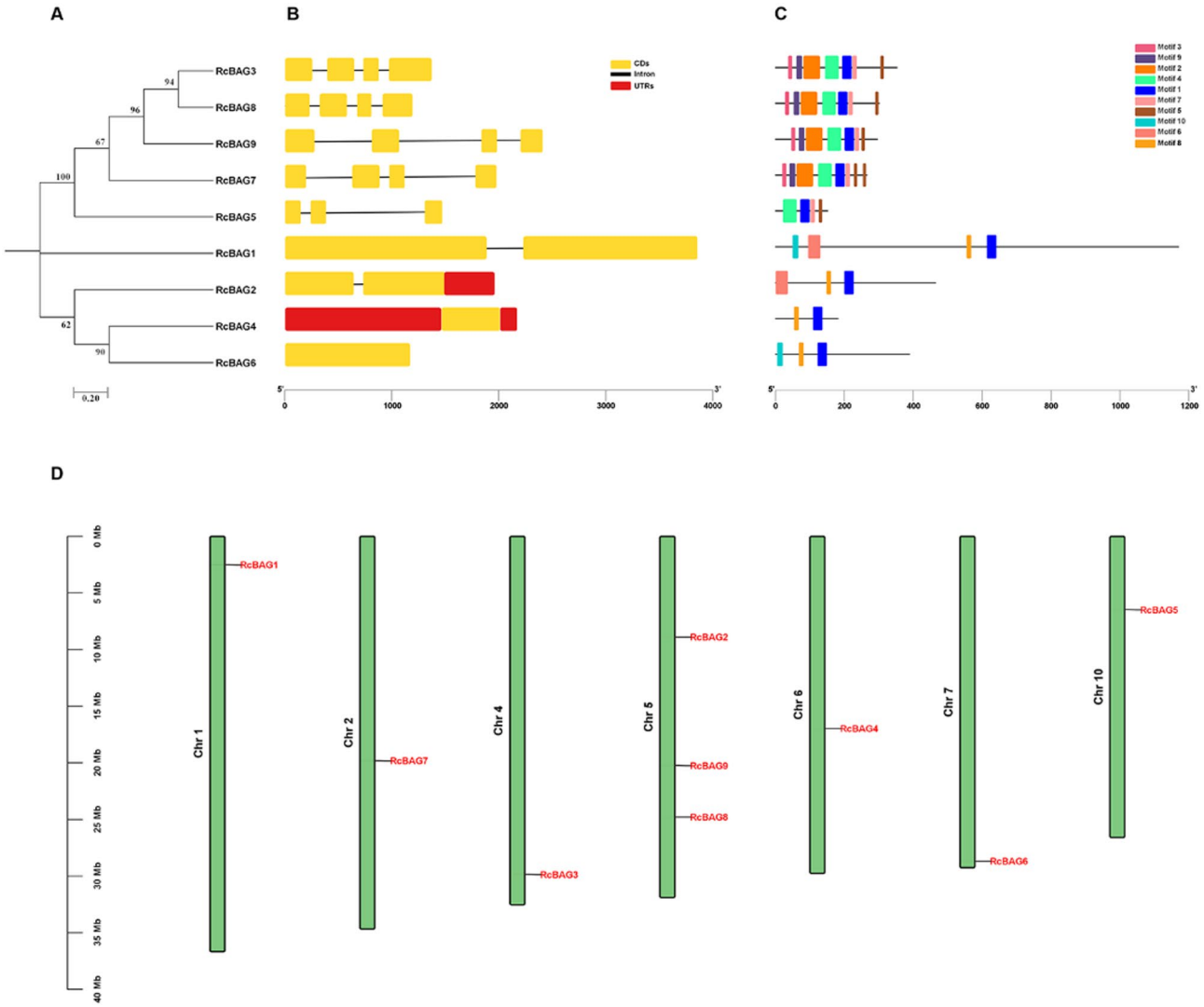

Fig. 1. *RcBAGs* structural and chromosomal location analysis. (**A**) Phylogenetic tree created using the MEGA software, utilizing the full amino acids sequences of 9 RcBAG proteins. (**B**) *RcBAG* gene exon and intron structures. The CDS and UTR portions are depicted as yellow and red rectangles, respectively, while the intron is represented by a black line. The CDS, UTR, and intron lengths of *RcBAGs* are displayed in a proportional manner. (**C**) Different colored boxes represent conserved motifs discovered in RcBAG proteins. Using MEME Suite 5.4.1 (https://meme-suite.org/meme/) ten conserved motifs were found. (**D**) Chromosomal locations of all the 9 *RcBAG* genes.

domain; RcBAG4 and RcBAG6 have the IQ domain as well as the BAG domain, whereas RcBAG3 contains Ubiquitin (UBL) like domain along with BAG domain (Fig. 2).

Collinearity and gene duplication analysis

Genomic duplications have long been thought to be crucial for the emergence of evolutionary innovations. According to research, duplicate genes arise at a high rate, around 0.01 per gene every million years. However, most of these copies are silenced or suppressed within a few million years; those that remain are subsequently exposed to rigorous purifying selection [63,64]. Plants can have either large-scale genomic duplications (WGD) or small-scale duplications such as tandem and segmental duplications [64,65]. Studying duplication event of *BAG* genes across different species helps better understand evolutionary relationships. To better speculate the conservancy and importance of these genes, synteny analysis was carried out with 4 crops belonging to same family, clade, and class. This analysis revealed about 40 syntenic pairs. Among these 12 gene pairs were found between *R. communis* and *P. trichocarpa*, 15 between *R. communis* and *M. esculenta*, 7 pairs between *R. communis* and *S. lycopersicum*, and 6 pairs between *R. communis* and *A. thaliana*. Maximum pairs were identified in *M. esculenta*, and *P. trichocarpa* which is indicative of shared evolutionary history among these three crops (Fig. 3). Additionally, duplicated gene pairs nonsynonymous (Ka) and synonymous (Ks) substitution rates were computed. Ka and Ks values are thought to be significant indicators for examining the selective pressure or

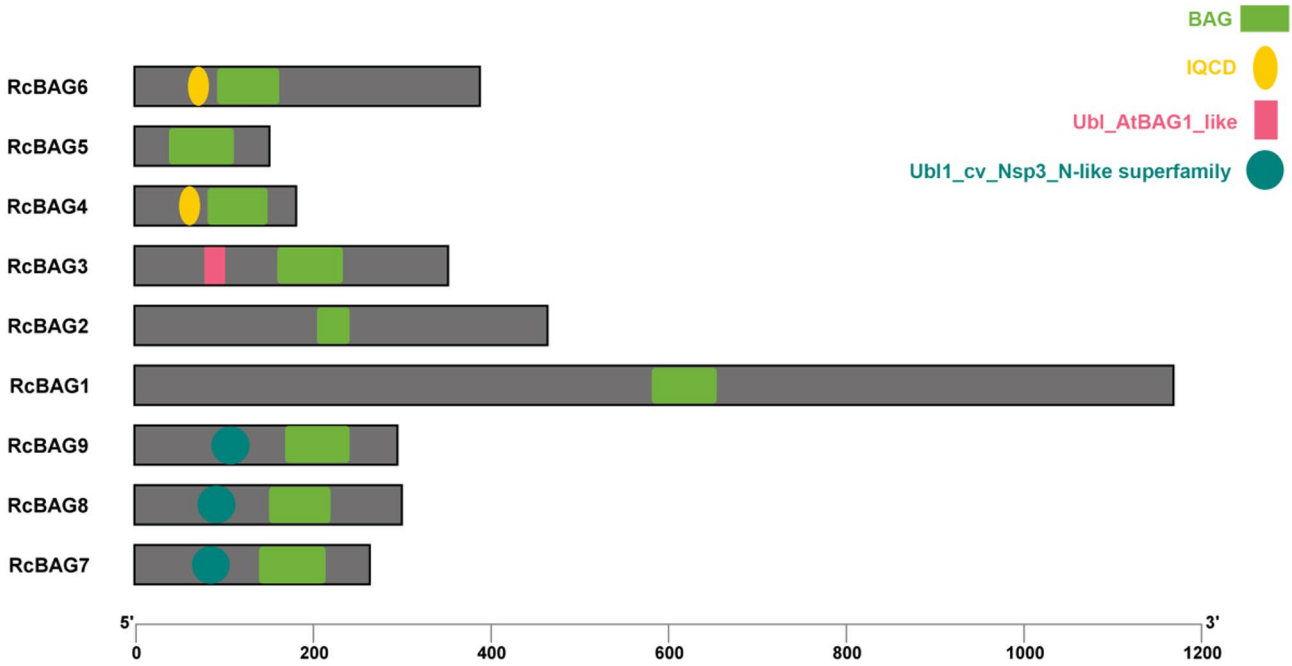

Fig. 2. Advanced genome schematic representation of conserved 9 *BAG* genes of *Ricinus communis* having BAG domains. The bottom scale can be used to calculate the length of the RcBAGs protein.

strength on a protein-encoding gene as well as for approximating the date/s of duplication events [64,66]. Further validation of functional conservancy and selection pressure was established through Ka/Ks analysis. Ka/Ks > 1 is indicative of natural or positive selection, Ka/Ks = 1 indicates neutral selection, while Ka/Ks < 1 is indicative of purifying selection. In case of BAG gene pairs, all values showed were less than 1, which demonstrated highly purifying selection across multiple species (**Table S3**). This indicates the functional and structural conservation of *BAG* genes across different crops and determined its essential evolutionary ties.

Evolutionary analysis of selected *BAG* genes in *R. Communis* L.

Functional difference among the many kingdoms of life can be better understood by looking at the evolutionary description of gene families. To conduct a phylogenetic study of the BAG-domain proteins in *R. communis*, eighty amino acid sequences were gathered from several plant species, including *R. communis*, *Arabidopsis thaliana*, *Cicer arietinum*, *Linum usitatissimum*, *Lupinus angustifolius, Trifolium pretense, Medicago truncatula, Glycine max, Phaseolus vulgaris, Vigna radiate, Vigna unguiculata, Lotus japonicas, Manihot esculenta, Lupinus albus, Populus trichocarp* and *Vitinus vinifera*. The highest likelihood technique was used to determine the evolutionary history. A bootstrap consensus tree based on 1000 iterations was used to demonstrate the evolution of the species under consideration. According to our findings, monocot and dicot plants exhibit a high level of protein conservation in their BAGs. Based on evolutionary investigations, RcBAGs have been classified into seven major groups (group's I–VII, Fig. 4). The most proteins are found in group II (RcBAG3, RcBAG5, RcBAG8, RcBAG9), followed by all others groups having one RcBAG proteins like group I (RcBAG7), group III (RcBAG1), group V (RcBAG4), group VI (RcBAG6), group VII (RcBAG2) and group IV having no RcBAGs protein respectively (Fig. 4). The greatest evolutionary relationship among BAG proteins was discovered between RcBAG1, RcBAG2, RcBAG6, and RcBAG4, followed by RcBAG9, RcBAG3 and RcBAG8 showing highest evolutionary resemblance, while RcBAG7 as out-group.

In Silico promoter analysis of *RcBAGs*

The Castor bean genome sequence database was utilized to discover the cis-acting regions that regulate *RcBAG* function. All *RcBAGs* 1 kb upstream area from the start codon has been found. PlantCARE performed an in-silico investigation on these promoters. The findings showed that these promoters contain a variety of cis motifs. Based on their respective functional activities, the identified cis-acting elements were categorized as stress-related elements, hormone response elements, and light response elements (Fig. 5A). The majority of *RcBAGs* promoters contain stress related and hormonal response elements, implying that they may be involved in the stresses. *RcBAG1*, *RcBAG2*, *RcBAG3* and *RcBAG4* promoters contain binding sites for MYB transcriptional factors (MBS), which govern the stress response. Aside from *RcBAG1*, *RcBAG2*, *RcBAG6*, *RcBAG8* and *RcBAG9*, we found another stress-responsive motif, the TC-rich repeat, in the majority of *RcBAGs* promoters. The promoter regions of *RcBAG* genes contain a number of motifs related to hormone signaling and regulation. Salicylic acid (TCA) was the most common motif in three *RcBAG* promoters, whereas ABA-responsive elements (ABREs) appeared in five. Other hormonal response elements that have been discovered include methyl jasmonate (MeJA) responsive elements (CGTCA-motif), ethylene response elements (EREs) in one RcBAG promoter, salicylic

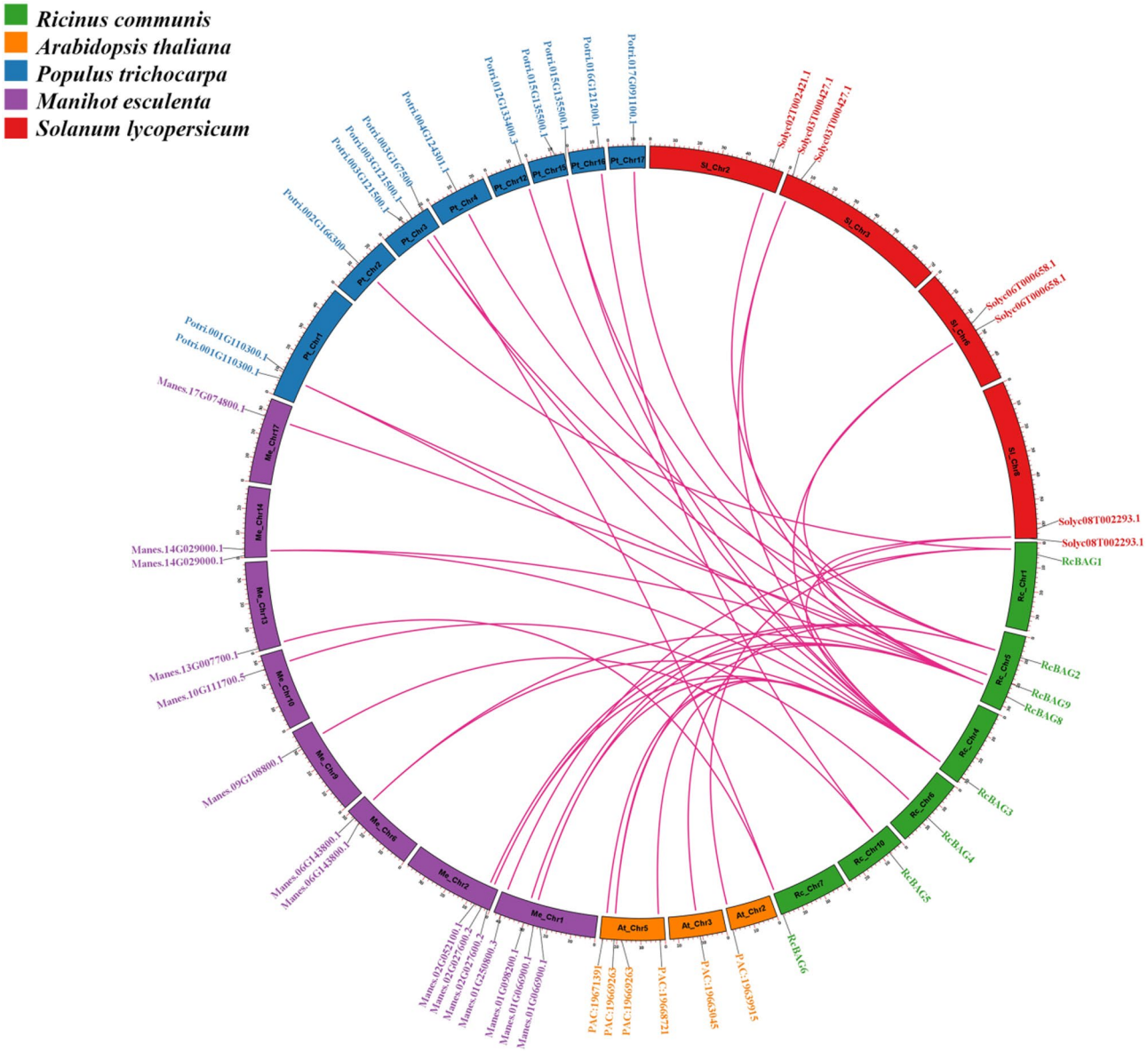

Fig. 3. Synteny investigation revealing the gene duplication of 9 RcBAG genes in Ricinus communis, Arabidopsis thaliana, Populas trichocarpa, Manihot esculenta and Solanum lycopersicum.

acid-responsive elements (TCA), auxin response like TGA element, AuxRR-core and gibberellin-responsive elements (GARE, TATC, P-Box) (Fig. 5A). Except these elements, some promoters like *RcBAG3* contain LTR elements which showed that *RcBAGs* may be involved in temperature responsiveness. Almost all promoters contain light responsive elements (GT1, TCT motif, Box4, TCCC motif, G-Box, GATA-motif, BoxII) except *RcBAG1* (Fig. 5A). Based on this analysis, *RcBAG* genes may play a role in hormone signaling, plant growth, and stress response.

Recently, the completion of genome sequences has enabled genotype and reference sequence comparisons for sequence analysis. As a result of this investigation, several genotypes with advantageous features were discovered. Furthermore, it serves as the foundation for more recent research, such as structural variation [67], which examines chromosomal variations to establish which alterations are produced by certain genes. As a result, all nine RcBAG protein sequences were investigated in the current work. The results revealed that BAG domain is highly conserved in all the RcBAGs studied (Fig. 5B). According to studies, hormones regulate this incredibly dynamic acetylation in plants, allowing them to adjust to a range of stress conditions [68]. The *RcBAG* family genes highly conserved sequence may explain their diverse activities in stress regulation and castor growth.

Interaction of RcBAG with other proteins

An in-silico protein–protein interaction (PPI) analysis of RcBAG proteins was performed using STRING v11.0, one of the most widely used PPI databases, which contains interaction data for 2,031 species.This database

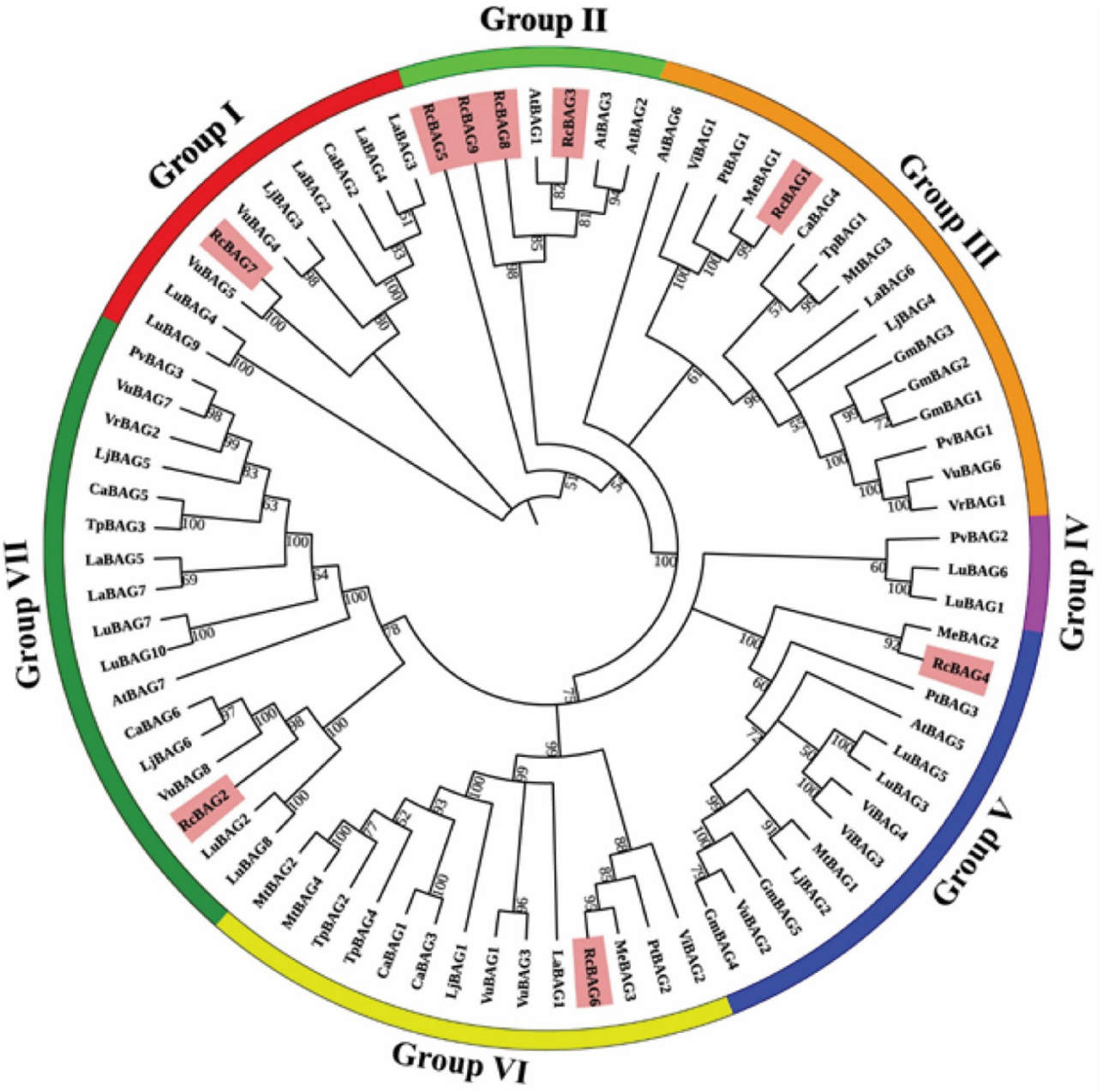

Fig. 4. A phylogenetic tree of the whole RcBAGs protein discovered in the genomes of castor and other legume plants. The (BAG) members protein sequences from *R. communis*, *Arabidopsis thaliana*, *Cicer arietinum*, *Linum usitatissimum*, *Lupinus angustifolius, Trifolium pretense, Medicago truncatula, Glycine max, Phaseolus vulgaris, Vigna radiate, Vigna unguiculata, Lotus japonicas, Manihot esculenta, Lupinus albus, Populus trichocarp* and *Vitinus vinifera* were brought into the MEGA X 10.1 (Molecular Evolutionary Genetics Analysis tool) software (https://www.megasofware.net/) and phylogenetic tree was constructed using maximum likelihood and bootstrap analysis with 1000 replicates/iterations.

uses co-expression data and experimental evidence to build global predictions regarding protein functional relationships. The protein–protein interaction map was generated using the indicated gene interaction/combination score threshold of 0.7 (Fig. 6). The interaction map inferred that RcBAG1, RcBAG3, RcBAG5, RcBAG7, RcBAG8, and RcBAG9 have strong protein–protein interaction with many HSP70 proteins (XP_002535157.1; XP_002528199.1; XP_002533498.1; XP_002519994.1; XP_002522946.1) (Fig. 6). Surprisingly, many RcBAGs interact with one another to form a complex with HSP70. Furthermore, no HSP70 proteins in the PPI map interacted with RcBAG2, RcBAG4, and RcBAG6 (Fig. 6).

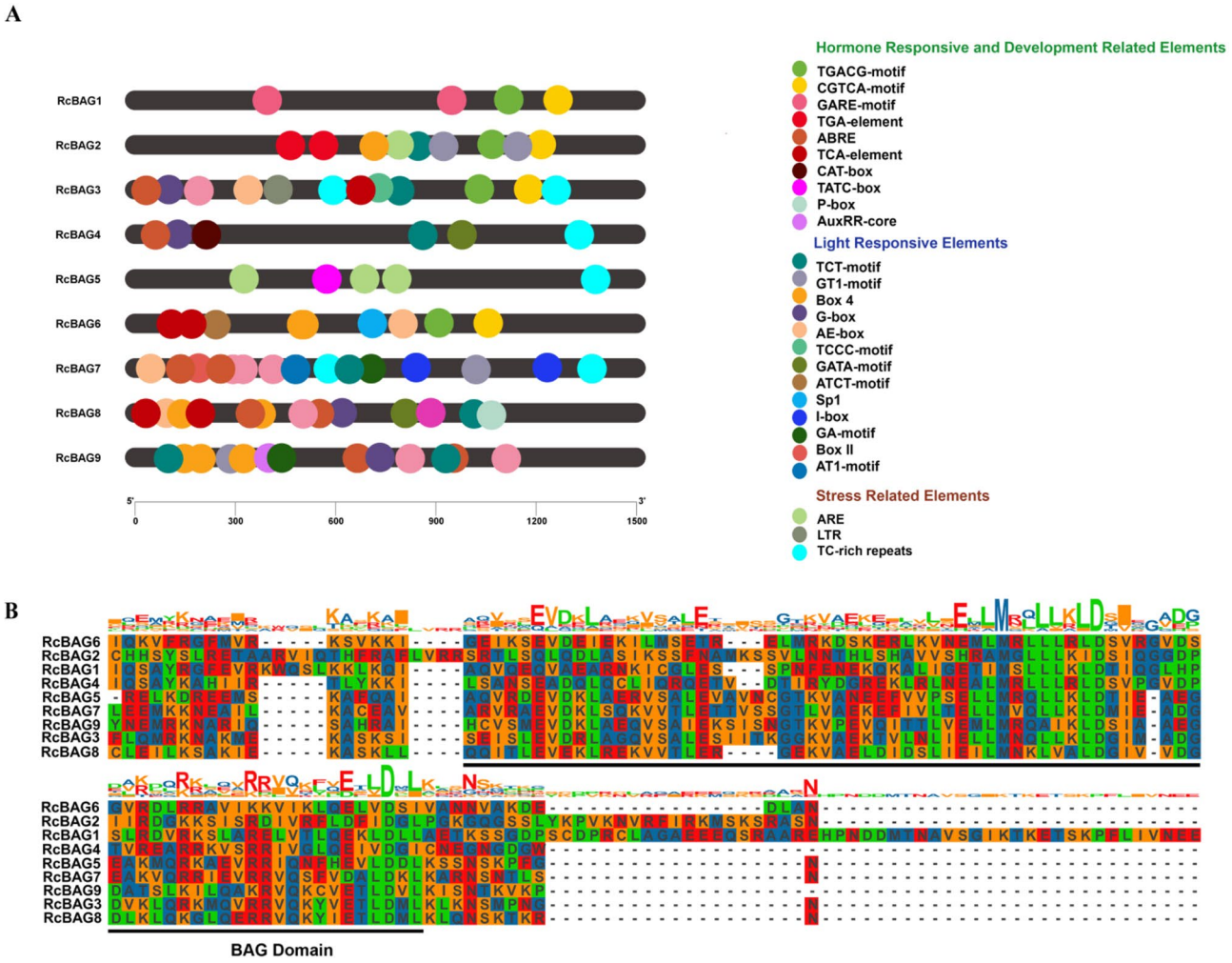

Fig. 5. Cis regulatory elements and sequence alignment analysis of *RcBAG* members in *Ricinus communis*. (**A**) Cis-acting regulatory analysis for 9 *RcBAG* genes, divided into three categories (Hormone, light and stress response) showed with different colors. (**B**) Multiple sequence analysis of the RcBAG proteins have been aligned, revealing conserved amino acid residues. The black line represented the BAG domain.

Expression analysis of *RcBAGs* at different developmental stages and responses to abiotic stresses

To learn more about the biological role of this family genes, qRT-PCR was utilized to assess the spatial expression of *RcBAGs* (using the primers listed in **Table S1**). The most of genes were elevated highest in seeds, with the exception of *RcBAG1* and *RcBAG4*, which demonstrated a small reduction in transcript accumulation (Fig. 7). The majority of *RcBAG* genes were also downregulated in stems and flowers. *RcBAG7* and *RcBAG3* expression was more significant in the seed than in the other genes. The accumulation of *RcBAG1* and *RcBAG9* transcripts was also increased in the young leaf, root, and seed, respectively (Fig. 7). Similarly, in *RcBAG2*, *RcBAG4* and *RcBAG8*, seed expression is downregulated. Comparing stem and old leaf, the expression of *RcBAG3*, *RcBAG4*, *RcBAG6*, *RcBAG7* and *RcBAG9* were decreased. The expression of *RcBAG4* was deficient in almost all tissues tested (Fig. 7).

The promoters of *AtBAG* and *SlBAG* genes include a number of stress-related cis-acting elements, and previous studies on *Arabidopsis* and tomato BAG proteins showed that they play a role in stress response [34,36]. For this purpose, we analyzed in silico promoters of *RcBAG* genes, and found that most *RcBAG* genes contain stress-regulating cis-elements such as MYB transcriptional factor binding sites (MBS), TC-rich repeat, ABA-responsive elements (ABREs), salicylic acid (TCA), MeJA responsive elements (CGTCA-motif), ethylene response elements (EREs) auxin response like TGA element and AuxRR-core and gibberellin-responsive elements (GARE, TATC, P-Box). Previously reported that the majority of *AtBAGs* and *SlBAGs* were involved in either cold stress response or heat stress because of their direct interaction with HSPs proteins or indirectly with HSPs through other functional partners [35,40–42]. So, the expression of *RcBAG* family genes was examined using qRT-PCR under heat and cold at different time intervals [35,36,61]. All *RcBAG* genes were upregulated in response to cold stress when seedlings were exposed to cold stress (4 °C) for 6, 12, and 24 h (Fig. 8). With the exception of *RcBAG8,* this showed down-regulation at six h and 24 h but showed two-fold upregulation after 12 h cold

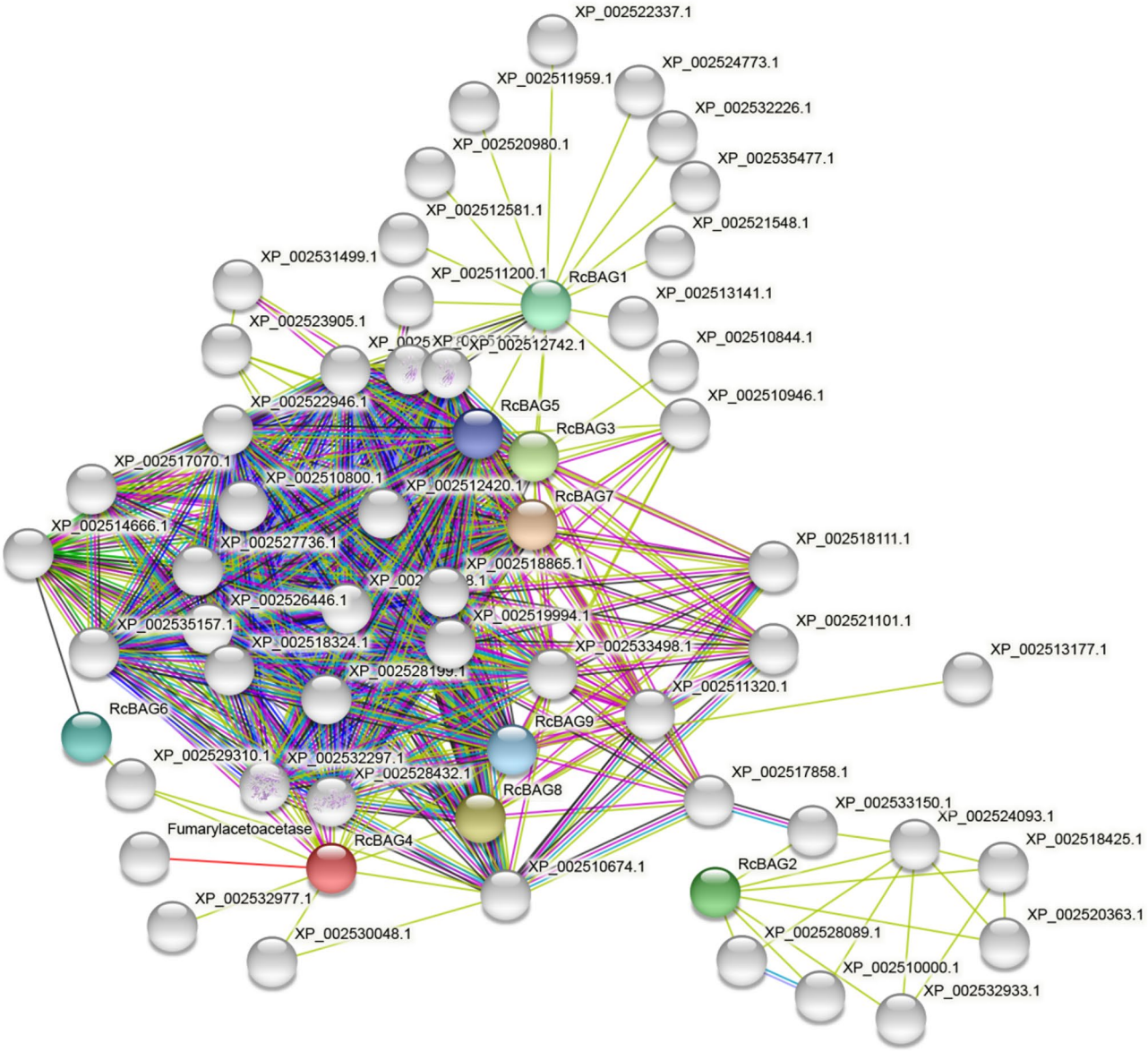

Fig. 6. Analysis of RcBAGs interactions with other proteins. Predictive PPI networks utilizing caster bean RcBAG proteins were acquired from STRING (https://string-db.org/). Gene fusion evidence is shown by red lines, neighborhood evidence by green lines, blue lines showed co-occurrence evidence, experimental evidence by purple lines, yellow lines showed text mining evidence, database evidence by light blue lines, and coexpression evidence by black lines.

treatment respectively. Similarly, *RcBAG9* expression is decreased only at six h but upregulated at 12 and 24 h of cold treatment (Fig. 8). Notably,all *RcBAG* genes increased their expression in response to cold stress, although at different periods. *RcBAG1* and *RcBAG4* showed the most significant expression at the start (6 h), followed by 12 and 24 h, respectively (Fig. 8). These findings suggest that *RcBAGs* can modulate cold stress signaling in addition to being implicated in cold stress tolerance.

Previous literature about *BAG* genes in *Arabidopsis*, tomato and other species suggested that it is involved in heat stress tolerance. Analysis of the cis-acting components of the *RcBAG* gene promoters revealed many cis-acting sites associated with abiotic stress response. To further check the expression of nine *RcBAG* members in response to heat stress, we treated castor seedlings at 37 °C at different time intervals [26,61]. Results revealed that almost all genes showed increased expression at 37 °C at different time points except *RcBAG7* at three h and six h. In comparison, *RcBAG9* at one h and 3 h respectively (Fig. 9). Following prolonged heat stress (37 °C), *RcBAG2*, *RcBAG4*, and *RCBAG6* expressions showed a rising trend (at one h and 3 h) compared to control, but decreased at 6 h (Fig. 9), while *RcBAG7* expression decreased both at 3 h and 6 h respectively (Fig. 9). These data showed that *RcBAGs* may be crucial in heat stress tolerance by possibly interacting with HSPs proteins.

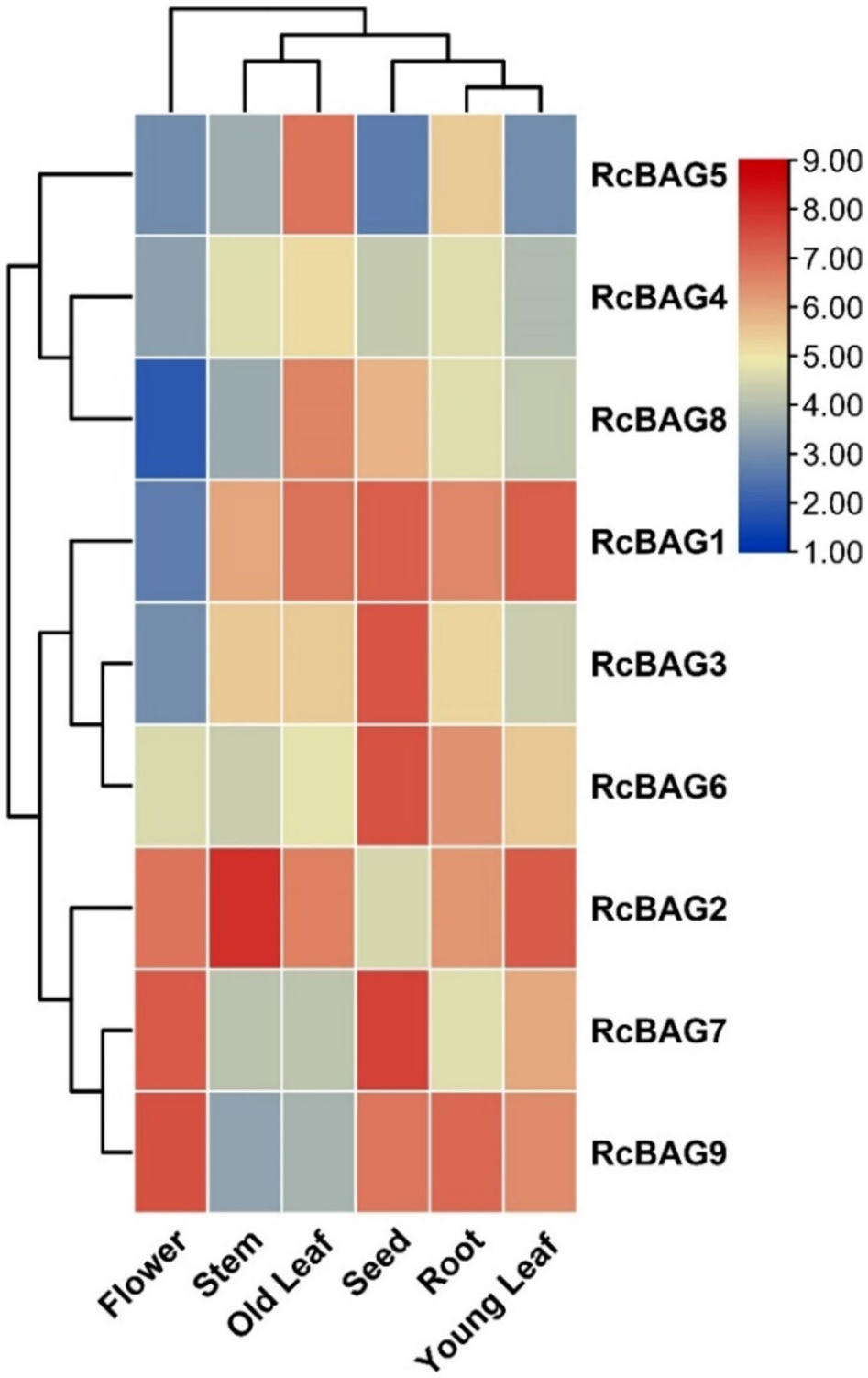

Fig. 7. The expression of *RcBAG* genes in various organs was investigated using qRT-PCR. Using actin as an endogenous control, qRT-PCR was used to investigate the transcript accumulation of *RcBAGs* in diverse tissues. Using the TB tool, a heat map showing gene expression was generated from qRT-PCR results.

Discussion

All microbes, plants, and mammals have proteins with BAG domains. Though knowledge of BAG in plants is severely limited, the most studied BAG proteins are those from rice, maize, tomato, and soybean [34–36,46,51], as well as the Arabidopsis BAG family [27,61]. Arabidopsis BAG proteins are primarily responsible for regulating plant developmental processes and stress responses. We discovered and carefully examined BAG domain-containing

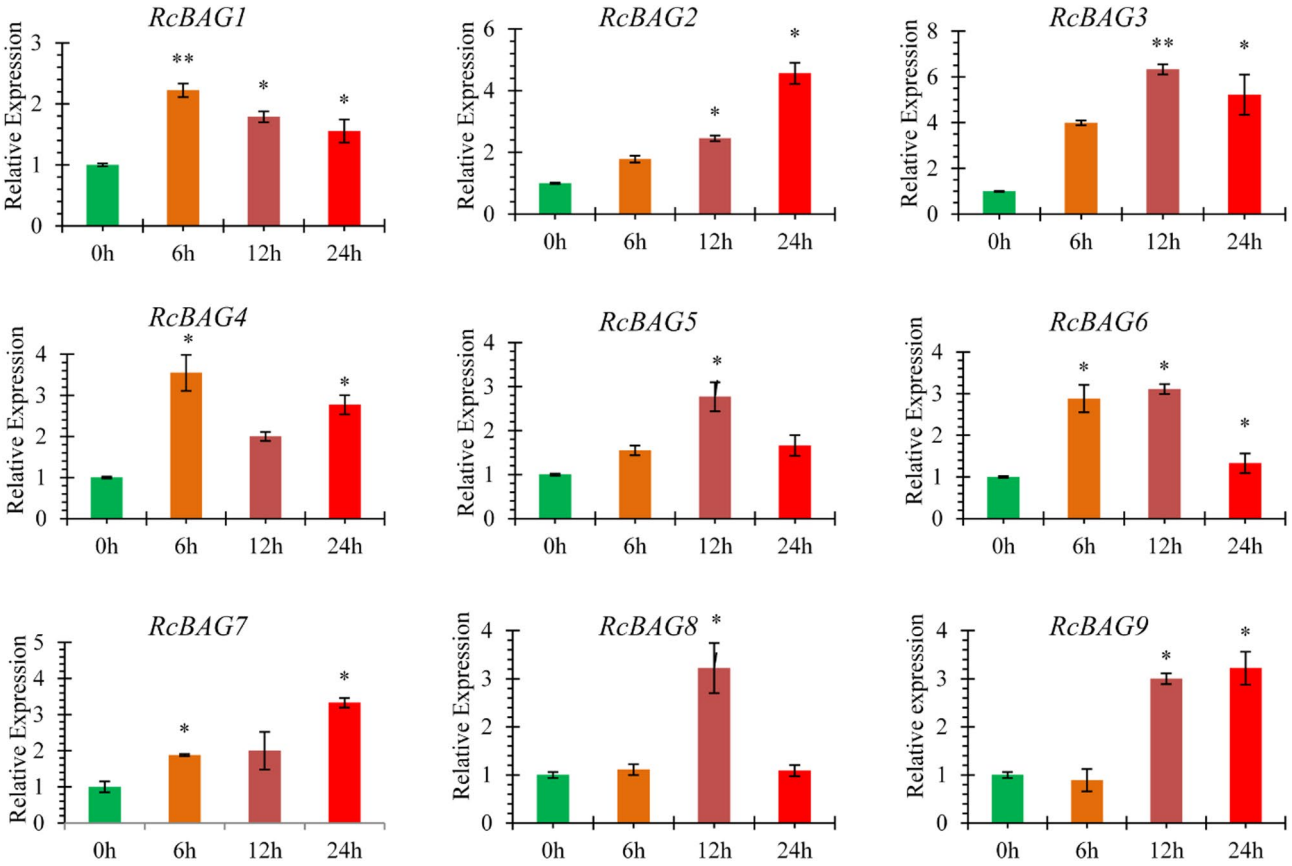

Fig. 8. The expression profile of *RcBAGs* during cold stress (4 °C) was investigated using qRT-PCR. Actin served as an endogenous control, and reference expression level 1 was used to assess transcript accumulation in 21-day-old castor seedlings treated with 4 °C cold stress by qRT-PCR at 6-, 12-, and 24-h intervals. Asterisks represents standard error of three biological replicates, assessed using the student's t-test (*$p < 0.05$, **$p < 0.01$).

proteins in castor bean (*R. communis* L.) using bioinformatics and wet-lab molecular biology approaches. The castor bean has nine BAG proteins, whereas *Arabidopsis* only has seven, indicating that it is a more evolutionary complex species. In addition to castor beans, BAG proteins have been identified in a variety of other legume plants, including *Cicer arietinum*, *Linum usitatissimum*, *Lupinus angustifolius, Trifolium pretense, Medicago truncatula, Glycine max, Phaseolus vulgaris, Vigna radiate, Vigna unguiculata, Lotus japonicas, Manihot esculenta, Lupinus albus, Populus trichocarp* and *Vitinus vinifera*.

Based on phylogenetic study, BAG proteins are shown to be highly conserved across the plant species. Seven classes of castor bean BAG-domain proteins have been identified by evolutionary study (Fig. 4). Based on a phylogenetic analysis, the majority of *RcBAG* genes were shown to be more closely related to BAG proteins present in other species. The results show that plant BAGs referring to the same categorized group might fulfill comparable tasks. BAG genes have previously been identified in many crops and animals. Due to the tissue-specific expression, stress responsiveness, and PCD, these genes are found to be highly conserved across multiple organisms. We carried out synteny analysis for our identified 9 *BAG* genes to find correlation with other crop relatives and better understand the evolution of these genes. We identified seven gene pairs in *Arabidopsis*, and these genes were found to be on chromosomes 2, 3, and 5, which correlated with a previous study [27]. Similarly, *BAG* genes were found on Chr 2, 3, 6, and 8 for *S. lycopersicum, which* also correlates with previously identified *SIBAG* genes [29]. More pairs were found on *M. esculenta* and *P. trichocarpa* as they are more closely related to *R. communis*. All of these syntenic pairs showed great purifying selection, which indicates the importance of *BAG* genes and how their function and structure remain conserved despite evolution and significant species differences.

Further functional characterization may reveal more about how *RcBAG* genes respond to various forms of stress, such as pathogen hypersensitivity and abiotic stressors, such as salt, drought and UV radiation. Domain analysis utilizing the (MEME) software was used to investigate the functional diversity of RcBAG proteins. This showed that all RcBAGs share a single, highly conserved BAG domain. The N-terminal UBQ domains of several RcBAG proteins, including as RcBAG3, RcBAG7, and RcBAG9, are comparable to BAG proteins from other species. Stress-induced autophagy and ubiquitin–proteasome degradation are hypothesized to be mediated by BAG proteins with UBQ domains identified in *Arabidopsis* and humans [27]. Calcium (Ca^{2+}) stimulates two BAG genes with an *Arabidopsis* calmodulin-binding motif [27]. Several earlier studies have demonstrated that these IQ calmodulin-binding motif BAG proteins, which are involved in Ca^{2+} signaling during PCD and plant stress

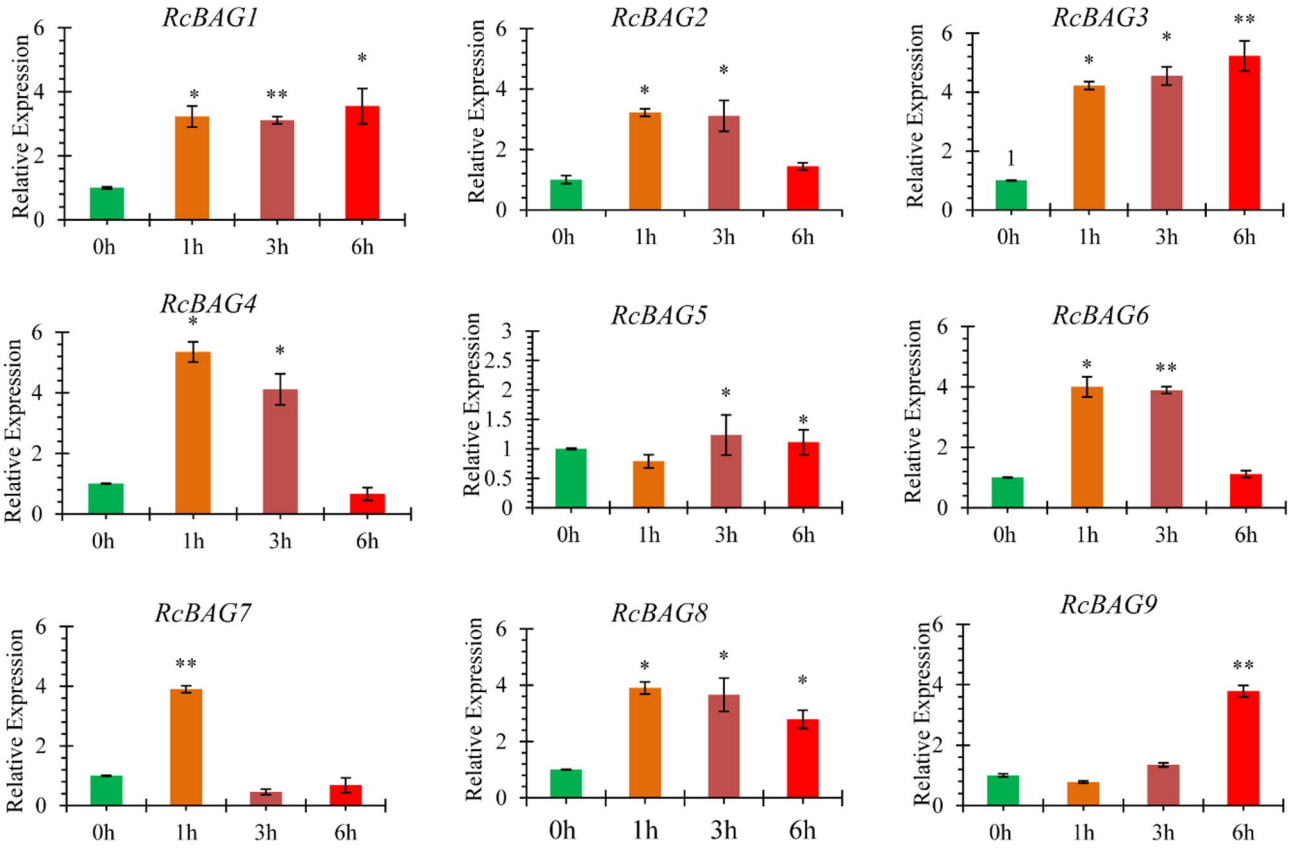

Fig. 9. qRT-PCR was used to evaluate the expression profile of *RcBAGs* under heat stress (37 °C). Actin was employed as an endogenous control to assess transcript accumulation in 21-day-old castor seedlings subjected to 37 °C heat stress. The reference expression level 1 was examined at 1-, 3-, and 6-h intervals. The results (*$p < 0.05$, **$p < 0.01$) represent the mean (± SE) of three biological replicates. The data is evaluated with the student t-test.

responses, are regulated by Ca^{2+} and control plant senescence [69] and in castor beans identified *BAGs*, *RcBAG4* and *RcBAG6* containing IQ motif domain, which probably involved in Ca^{2+} signaling pathways to regulate stress responses.

In order to enhance understanding of transcriptional regulation, the promoters of *RcBAGs* for cis-acting regions were investigated in silico. *RcBAGs* are involved in plant stress response via a variety of stress-responsive components, including (such as MBS, LTR, TC-rich repeats, ABRE, ERE, GARE, and the CGTCA motif). Research on *BAG* genes from Arabidopsis and other species, which contain same stress response components is consistent with these findings [34,36,44,47,48,61]. Furthermore, it has been proven that these stress-related components are involved in *BAG* gene regulation during plant stress response due to increased β-glucuronidase (*GUS*) activity generated by the *AtBAG2* and *AtBAG6* promoters under salt and osmotic stress, as well as following 1-aminocyclopropane-1-carboxylic acid (ACC) and ABA treatment [26]. The presence of stress-related elements in BAG promoters may be related to *BAG* genes stress-specific responses, as *BAG* genes from many plants, including *Arabidopsis*, rice, soybean, and wheat, have demonstrated plant-specific responses and have been successfully used to improve stress tolerance in *Arabidopsis* and rice [26,51,70]. *RcBAGs* varied expression patterns across tissues demonstrate their potential role in plant stress response (Figs. 7, 8, 9). Under salt stress conditions, *Arabidopsis* homologues of these genes (such as *BAG2*, *BAG3*, *BAG7*, and *BAG6*), showed increased expression [26,27,61]. Cold temperatures increased *AtBAG4* expression in Arabidopsis, while transgenic tobacco plants overexpressing *AtBAG4* demonstrated cold stress response [27]. Furthermore, the great majority of *RcBAG* genes showed varied expression patterns in different tissues (Fig. 7), indicating that *RcBAGs* play a role in plant development and growth.

An essential resource for understanding the functional differentiation of gene families in plant tissues and organs is tissue-specific expression profiling. To elucidate the *BAG* genes expressions in *R. communis*, we used qRT-PCR analysis of *RcBAG* genes at various phases of development. The findings demonstrate that *RcBAG* gene expression patterns vary throughout different stages of castor bean development. It's worth noting that most *RcBAG* genes are expressed differently in seeds, which may influence traits exclusive to castor bean seeds. Doukhanina et al. discovered a *BAG* EST (AI960691 or Glyma.01G123300.1) in soybean immature seed coat, which lends credence to this idea. In terms of evolution, the *BAG* in soybean is closer to *SIBAG8*, which also showed seed-specific expression [27,34].

Protein subcellular localization research contributes to the discovery of protein biological functions. RcBAG proteins were discovered in a range of subcellular sites in this investigation. Proteins like RcBAG1, RcBAG4, RcBAG6, RcBAG8, and RcBAG9 are usually present in the nucleus and cytoplasm, whereas RcBAG2, RcBAG3, RcBAG5 and RcBAG7 are located in the chloroplast. Previous studies have shown that *Arabidopsis* BAG proteins are present in a variety of subcellular locations, with AtBAG1-3 found in the cytoplasm and AtBAG4 identified in the cytoplasm with nucleus [27,71]. Similar to previous *Arabidopsis* studies, AtBAG5 and AtBAG6 have been demonstrated to localize in mitochondria and vacuoles to govern organelle-regulated cell death [69,72,73]. *RcBAG4* punctate expression was also found, indicating that a similar mechanism of *SlBAG4* activity may exist in tomato and *Arabidopsis* to regulate intracellular cell death [34]. A recent study has shown that the unfolded protein response (UPR) pathway, also known as the ER-nucleus stress-signaling system, is controlled by the translocation of ER-localized AtBAG7 to the nucleus in response to heat and cold stress [74]. *RcBAGs* may regulate the UPR pathway via interacting with HSP70 during stress, as some of these genes showed different expression levels under heat and cold stress.

Because BAG domains interact with HSP70/HSC70 proteins to modify chaperone activity, we performed a protein–protein interaction network analysis of RcBAGs to learn more about their activities and the molecular processes that drive their stress response. Many RcBAG proteins, including RcBAG1, RcBAG3, RcBAG5, RcBAG7, RcBAG8, and RcBAG9, interact with many HSP70 proteins, according to the interaction map. Other BAG proteins, such as (RcBAG2, RcBAG4, and RcBAG6), interact with other BAGs that bind with HSP70 but not directly with HSP70. This shows that in order to form groups with receptor molecules and cellular chaperones, RcBAG2, RcBAG4, and RcBAG6 interact to HSP70. The results of all of these PPI studies indicate that BAG domains enable BAG-family proteins to operate as adaptor molecules by enlisting HSP70/HSC70 to modify the pathways involved in plant growth and stress response, as well as target protein activity. Therefore, using *RcBAG* gene ectopic expression in combination with *HSP70* in plants might be a unique way to give plants stress tolerance for long-term sustainable agriculture. Castro et al. [47] applied a similar strategy to uncover *BAG* genes in *Physcomitrium patens* and showed that several members of the *P. patens BAG* gene family may influence heat responses, autophagy, and pathogen defense. To improve tomato cold resistance and photosystems and antioxidant systems, BAG8 interacts with PP2A, which regulates stomatal growth, and Hsp70, which regulates photosynthesis [41]. HSP70, on the other hand, helps tomatoes achieve BAG9-mediated thermotolerance by preserving photosystem stability and increasing antioxidant system efficacy [42].

RcBAG expression patterns vary based on abiotic conditions, but consistent trends after cold and heat stress suggest similar reactions to the castor. Recently, in Arabidopsis and tomato, numerous abiotic stimuli, particularly heat and cold stress, have been shown to influence the expression of *BAG* genes [41,42]. The results suggested that this variation in gene expression might be due to direct or indirect interactions with HSP70 proteins or transcription factors such as WRKY. More research is needed to understand the effects of *BAG* genes in diverse species, particularly in industrial crops like as *R. communis*.

Conclusion

This study provides a comprehensive genomic investigation of the *RcBAG* gene family in *Ricinus communis* (castor). Nine *RcBAG* genes were identified and classified into seven groups based on phylogenetic relationships. Detailed analyses of their physicochemical properties, conserved domains, chromosomal locations, gene structures, synteny, and promoter elements suggest a complex evolutionary history and potential regulatory roles under stress conditions. The diverse expression profiles of *RcBAG* genes across various tissues and developmental stages, especially under cold and heat stress, highlight their potential involvement in stress adaptation mechanisms. Subcellular localization predictions further support their functional diversity, indicating roles in multiple cellular compartments. Importantly, several *RcBAG* genes demonstrated strong stress-responsive expression patterns, underscoring their potential utility in improving abiotic stress tolerance in castor and related crops. Future research could focus on functional validation of key *RcBAG* genes through gene editing tools such as *CRISPR/Cas9* and explore their interaction networks within stress signaling pathways. Additionally, the use of stress-inducible or tissue-specific promoters could aid in developing genetically enhanced castor varieties. Overall, this work lays a solid foundation for further functional characterization of *RcBAG* genes and their application in castor improvement programs.

Data availability

All data generated or analyzed during this study are included in the article and its supplementary information files.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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