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Genome-wide investigation of cytokinin oxidase/dehydrogenase (CKX) family genes in *Brassica juncea* with an emphasis on yield-influencing *CKX*

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Rapeseed mustard (*Brassica juncea*) is an important edible oilseed crop whose yield is impacted not only by the biotic and abiotic stresses but the low productivity of the cultivars also has a great impact over it. In the past, cytokinin oxidase/dehydrogenase (CKX) family gene manipulation has increased yield and stress tolerance and has been cloned and characterized in different plant species. Cytokinin oxidase/dehydrogenase (CKX) is an important enzyme regulating cytokinin homeostasis and regulating the yield and tolerance traits in plants. This study demonstrated a comprehensive investigation of the CKX gene family in *B. juncea* cv. Varuna. As a result, a total of 24 CKX genes were identified across the 36 chromosomes (AABB) and classified into seven distinct subgroups. These seven subgroups were annotated based on their homology with their counterparts present in *Arabidopsis thaliana*. Expression pattern analysis by RT-qPCR in the high-and low-yielding *B. juncea* cultivars showed that all 24 CKX expressed differentially in various tissues; most were expressed in leaves, stems and developing siliques. The two functional domains, FAD-binding and Cytokinin-binding domains required for CKX activity, were conserved across the CKX family genes. Our findings systematically revealed the evolutionary dynamics of the BjCKX family genes. They led the foundation for subsequent validation of the CKX for their functional role in yield enhancement in *B. juncea*.

Keywords *Brassica juncea*, CKX, BjCKX, Cytokinin oxidase/dehydrogenase, Oilseed, Cytokinin, Rapeseed-mustard

Abbreviations

CKX	Cytokinin oxidase/dehydrogenase
Bj	Brassica juncea
Ip	Iisopentenyl adenine
Z	Zeatin
DZ	Dihydro zeatin
PJK	Pusa Jaikisan
Kb	Kilobase
Bp	Base pairs
DAS	Days after sowing
FAD	Flavin adenine dinucleotide
CDS	Coding sequence
IP	Isoelectric point
CRE	Cis-regulatory element

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Rapeseed-mustard is a widely cultivated oilseed crop with high economic value. India contributes 13% of the total global production of mustard, with approximately 117 lakh tons, and ranks as the fourth largest rapeseed-mustard producer in the world. The production of crops is significantly affected by environmental stresses and the genetic makeup of the cultivars. For example, heat stress or high temperatures can reduce seed germination, the number of secondary and tertiary branches, silique density and siliques per plant, while drought stress and low cytokinin levels in reproductive tissues result in less growth and development, ultimately leading to lower crop production. Several studies have shown that the CKX gene family members have negative regulation over yield enhancement in various crops, including rice, wheat, and rapeseed. Therefore, the present study has taken up the genome-wide identification of the CKX gene family using whole genome sequences of *B. juncea* cv. Varuna, and conducted molecular analysis for the characterization, classification, structural diversification, genomic distribution, and identification of CKX genes putatively involved in yield enhancement.

Cytokinin is a key phytohormone influencing plants' growth and many physiological functions by regulating cell division, shoot and root growth, signal transduction, vascular development, shoot apical meristem, and seed development. Cytokinin is present in plants mainly in the form of isopentenyl adenine (iP), zeatin (Z), and dihydro zeatin (DZ)[1]. Cytokinin oxidase/dehydrogenase (CKX) catalyzes the irreversible degradation of cytokinin by cleaving the side chain of the purine ring and making the cytokinin inactive[2]. This enzyme plays an important catabolic activity in regulating cytokinin concentration and decreasing their accumulation. In the recent past, many functional studies have revealed the role of CKX, in regulating the cytokinin levels in plants by which it enhances the crop yield and stress tolerance. For example, overexpression of the *PpCKX1* enhances drought and salinity tolerance in moss[3], while downregulation of the *OsCKX2* in rice increases the resistance to salinity stress without affecting the yield[4]. Similarly, various CKX genes have been identified for their role in enhancing stress tolerance in several plant species including barley[5], Arabidopsis[6,7], Alfalfa[8], and chickpeas[9] under the drought, salinity, or high-temperature stresses. Moreover, CKX's involvement in biotic stress tolerance is evident from the influence of resistance to pests like the Brown Plant Hopper in rice[10], pathogens like *Plasmodiophora brassicae* in *B. oleracea*[11], and *Botrytis cinerea* in Arabidopsis[12]. Additionally, *CKX* expression affects nutrient uptake and accumulation in plants, such as phosphorus and minerals, making it a critical factor in optimizing plant growth and nutritional content in crops such as rice[13], maize[14], barley[5], and rapeseed (*B. napus*)[15]. The double mutants for *ckx3 ckx5* have shown an increased seed yield with more numbers of flowers, siliques, and branches as compared to wild-type Arabidopsis[16] and with improved seed weight in *B. napus*[17]. These findings suggested that plant CKXs are anticipated to be involved in multifaceted functions in many plant species and bear the potential for crop improvement with their genetic manipulation.

In recent years, genome-wide bioinformatics analyses of multigenic families, like cytokinin oxidase/peroxidase, have been greatly helpful in understanding the physiological roles and characteristics of the individual gene family members. The *CKX* gene family is widely distributed and it is constituted of varying numbers of members in different plant species including monocots like rice (*Oryza sativa*) contains 11 *CKX*[18]; wheat (*Triticum aestivum*), *35*[19] maize, *(Zea mays), 14*[20], barley *(Hordeum vulgare), 11*[21], Finger millet (*Elusine corocana*), 20[22], Foxtail millet (*Seteria italica*), 11[23] and the dicots like *Arabidopsis thaliana,* 7[24], *Brassica rapa,* 12[25], *Brassica oleracea,* 36[11], *Brassica napus,* 23[26], *Brassica juncea,* 23[27], 24[28], Soybean (*Glycine max*), 18[29], Chickpea (*Cicer arientinum*), 10[9], Common Bean (*Phaseolus vulgaris*), 10[9], *Medicago truncatula,* 14[30], *Jatropha curcas,* 7[31], Apple (*Malus domestica*), 12[32]. Genetic evidence has indicated that CKX is a family of enzymes responsive to biotic and abiotic stresses, nutrient uptake, and yield enhancement in several plant species.

Results

Characterization of *BjCKX* gene family

The nucleotide sequence alignment of the 23 *CKX* genes known in *B. juncea* var. Tumida with the whole genome sequence of *B. juncea* cv. Varuna by using the BLASTn program of BLASTN 2.13.0 + resulted in a staggered 1574 *CKX* homologous sequences with the e-value 0.01. From the staggered 1574 sequences, 112 sequences were filtered and unified based on the e-value, query coverage hit alignment, and by neglecting the redundancy in the sequences. Following filtration, and alignment 112 sequences were mapped to 24 distinct CKX loci on the *B. juncea* cv. Varuna chromosomes, as shown in Table 1 and henceforth were designated as *BjCKX*. All the identified *BjCKX* (BjCKX 1-BjCKX 24) (Genebank *accession numbers* BankIt2784537 BjCKX-1 to BjCKX-24 *as* PP102224 to PP102247) were classified into seven groups and subgroups based on their homology counterparts present in *A. thaliana* and physical position in the *B. juncea* cv. Varuna chromosomes. The individual *BjCKX* genes grouped in a single clade were named based on their phylogenetic grouping and the karyotypic order in which they present. For example, *BjCKX a.b*, where 'a' denotes to which the *BjCKX* placed together with the Arabidopsis *CKX* in phylogenetic clade and 'b' denotes the numeric order of chromosomes in the ascending order. Genes names in the groups were assigned in ascending order of the chromosome as a distribution made for the Arabidopsis *CKX* genes classified into 7 groups. These 24 *BjCKX* genes were found unevenly distributed over the A (n = 10) and B (n = 8) chromosomes of the amphidiploid *B. juncea* (n = 18) with the AABB genome as shown in Fig. 1. The predicted gene length and coding sequences of the *CKX* genes in *B. juncea* vary from 2250 (*BjCKX 1.4*) to 6651 (*BjCKX 1.6*) bp and 1515 (*BjCKX 2.3*) to 3720 (*BjCKX 3.3*) bp, respectively. Similarly, the predicted protein length and molecular weight of the CKX genes in the *B. juncea* vary from 504 aa/ 55.8064 kDa to 1239 aa/137.03789 kDa. The theoretical PI ranged from 4.97 (*BjCKX 7.2*) to 11.09 (*BjCKX 1.6*) while GRAVY (Grand Average of Hydropathy) ranged from – 0.822 (*BjCKX 1.6*) to 0.03 (*BjCKX 4.1*). Moreover, the in silico subcellular localization of all the BjCKX-predicted proteins revealed their compartmentalization mostly in the endoplasmic reticulum followed by vacuoles, and extracellular spaces. Vacuolar-localized BjCKX proteins exhibited pI values between 7 to 9 and those in ER had pI values between 5 to 7, and extracellular BjCKX proteins had pI values ranging from 5 to 5.6. Detailed information about the chromosomal distribution of the predicted *BjCKX* gene and their length, CDS, protein length, PI, MW, and other related descriptions are shown in Table 1.

Genes	Chromosome	Start site (bp)	End site (bp)	Strand	Gene length (bp)	CDS (bp)	Protein length (aa)	Molecular weight (kDa)	Isoelectric point (*pI*)	Subcellular localization (mPLOC)	GRAVY score
BjCKX1.1	A03	10422992	10425416	Plus	2425	1584	527	59.25854	7.76	Vacuole	-0.224
BjCKX1.2	A04	20937556	20940222	Plus	2667	1647	548	61.99089	9.1	Vacuole	-0.247
BjCKX1.3	A05	1361663	1365270	Plus	3608	1602	533	60.45011	8.82	Vacuole	-0.288
BjCKX1.4	B03	14489260	14491509	Plus	2250	1635	544	61.34903	8.4	Vacuole	-0.237
BjCKX1.5	B04	339050	341717	Minus	2668	1650	549	62.10906	8.8	Vacuole	-0.259
BjCKX1.6	B05	64372805	64379500	Minus	6651	2793	930	60.90831	11.09	Vacuole	-0.822
BjCKX2.1	A07	917304	922579	Plus	5276	1518	505	55.85684	5.63	ER	-0.05
BjCKX2.2	A09	6476127	6480811	Minus	4685	1782	593	64.98667	5.8	ER	0.015
BjCKX2.3	B06	13652090	13656076	Plus	3987	1515	504	55.80649	6.23	ER	0.024
BjCKX2.4	B07	1562041	1565905	Plus	3865	1518	505	56.09751	5.75	ER	-0.019
BjCKX3.1	A02	6604610	6607827	Plus	3218	1557	518	58.89508	5.62	ER	-0.212
BjCKX3.2	A10	11770140	11773292	Minus	3153	1521	506	57.69675	6.16	ER /Vacuole	-0.29
BjCKX3.3	B02	19561057	19567081	Minus	6025	3720	1239	137.03789	6.47	ER /Vacuole	-0.379
BjCKX3.4	B08	26776747	26779756	Plus	3010	1560	519	59.02426	5.88	ER /Vacuole	-0.21
BjCKX4.1	A03	28220712	28223865	Plus	3154	1575	524	59.02426	5.54	ER	0.03
BjCKX4.2	B08	3413840	3417110	Minus	3271	1575	524	58.18981	5.78	ER	0.016
BjCKX5.1	A07	26856891	26860334	Minus	3444	1575	524	58.83372	5.62	Extracellular	-0.256
BjCKX5.2	B07	29506000	29509157	Plus	3158	1590	529	59.38843	5.68	Extracellular	-0.238
BjCKX6.1	A09	40454533	40456793	Plus	2261	1539	512	59.38843	8.88	Vacuole	-0.201
BjCKX6.2	B03	67178381	67180622	Plus	2242	1632	543	61.31625	7.35	Vacuole	-0.25
BjCKX7.1	A02	4718383	4721854	Plus	3472	1554	517	57.5594	5.2	Extracellular	-0.154
BjCKX7.2	A10	14257495	14260720	Minus	3226	1578	525	57.91464	4.97	Extracellular	-0.127
BjCKX7.3	B02	23917282	23920874	Minus	3593	1605	534	59.22037	5.21	Extracellular	-0.126
BjCKX7.4	B08	23335693	23339222	Plus	3530	1569	522	57.82752	4.98	Extracellular	-0.139

Table 1. List of the *BjCKX* family genes of *B. juncea* cv Varuna genome with their genomic distribution, gene structure, protein features and their subcellular localization.

Phylogeny of *CKX* genes in *B. juncea*

To determine the homology and similarity among the BjCKX proteins and with those in *Arabidopsis*, a phylogenetic tree was generated with a bootstrap value of 1000 replicates. Based on the gene structure, the phylogenetic analyses showed that all the *CKX* were clustered into seven phylogenetic subclades, namely from I to VII (Fig. 2a). The large subclade I had 7 (6 BjCKXs and 1 AtCKX1), whereas, 5 (4 BjCKXs and 1 AtCKX) were present in each subclade II, III, and VII, respectively. Similarly, 3 CKXs (2 BjCKXs and 1 AtCKX) were found in subclades IV, V, and VI (Fig. 2b). The phylogenetic distribution revealed the diversification of the BjCKX proteins in *B. juncea* and based on the annotation of CKXs in *Arabidopsis*, BjCKX has also been annotated accordingly.

Structural analysis of *BjCKX* genes and proteins

For this purpose all the identified *BjCKX* genes were used for analyses of the exon–intron distribution and the presence of the conserved motifs and protein domains. The exon–intron analysis revealed that the number of exons in *BjCKXs* varied from 4 to 9 among all the phylogenetic clades. In contrast, *BjCKX* 3.1, *BjCKX* 3.2, and *BjCKX* 3.4 had five exons (Fig. 2). Among the *BjCKX* gene family members, most of them had 5 exons and 4 introns, except for some of the specific members like *BjCKX1.2*, *7.1*, *7.2*, and *7.4* in clad I and VII with 4 exons, BjCKX1.6, 2.2 with 8 exons in clad I and II, and *BjCKX*3.3 with 9 exons in clad III. Notably, the second exon with 128 bp was found conserved across the *BjCKX* except in the members of clad VII with 276 bp. Additionally, 3rd and 4th exons were observed to consist of varying lengths of 258, 261, 263, 267, and 269 bp. The observation has shown in general the CKX genes in the same subgroups show similar exon–intron features, supporting their phylogenetic relatedness (Fig. 3).

Furthermore, Motif analysis was performed as per the phylogenetic relationship using the MEME database. As a result, *BjCKX* that clustered in the same clad shared moreover common motif compositions, indicating functional similarities among members of the same *BjCKX* clade. The InterProScan annotation of the MEME motif analyses revealed that sixteen motifs (motifs 1–13, and 15–17) were noted as CKX protein motifs, those are characteristic features of the cytokinin oxidase/dehydrogenase gene family. Motif 14 was observed exclusively absent in the BjCKX clad II members, whereas, motif 18 was present exclusively in the clad I and VI members except BjCKX1.1 and BjCKX6.2. Similarly, motif 19 was exclusively present among the BjCKX clad III members and motif 20 exclusively in the clad II and clad IV members (Fig. 4a).

The domain analysis in BjCKX protein sequences revealed the presence of the two functional domains namely the cytokinin binding domain at the C-terminal and the FAD binding domain at the N-terminal in all the predicted BjCKX proteins from clad I to VII subgroups (Fig. 4b). In addition to these two conserved domains of

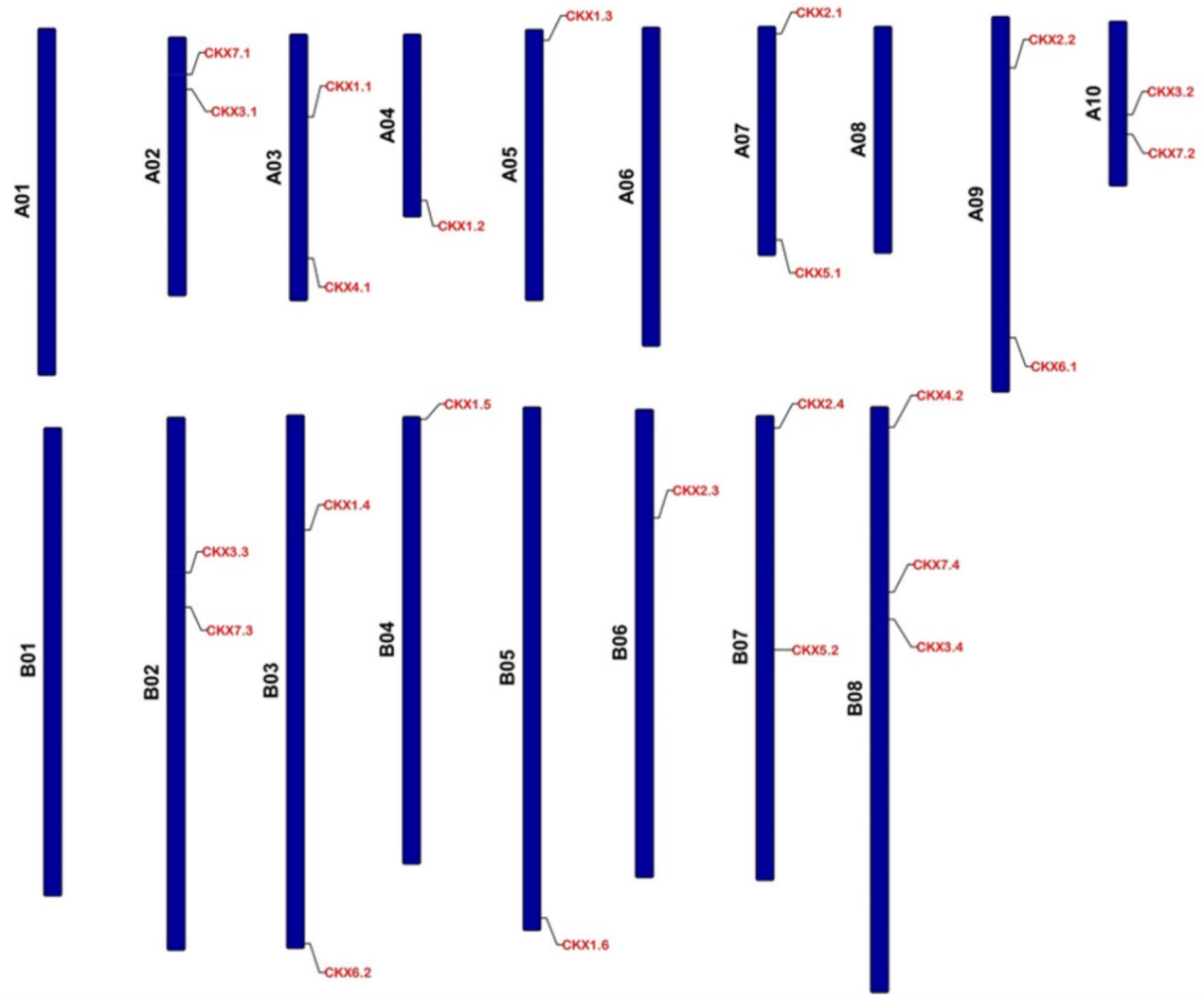

Fig. 1. Schematic representation of the *BjCKX* gene family members identified in *B. juncea* cv. Varuna on the A and B sets of chromosomes.

the CKX, BjCKX1.6 has the one dnaA (PRK14086) domain and BjCKX3.3 has the DUF4283 (cl16623) and AIR1 (COG5082) two additional domains which were present mostly in the post-translational modifying proteins.

Cis-regulatory element analysis in the promoter region of the *BjCKXs*

The cis-regulatory elements (CRE) present in the promoter region of a gene are primarily involved in regulation of the gene expression. We used 2 kb long promoter sequences upstream to the transcription start site (TSS) of all the *BjCKXs* to scan the CRE distribution using the PlantCARE database. Apart from the presence of the conserved core promoter elements viz. TATA- and CAAT-boxes, the other elements notably, light-responsive elements (ATC-motif, AE-box, Box II, Box 4, I-box, TCT-motif, Sp1, GATA-motif, GT1-motif), the phytohormone-responsive elements (ABRE, AuxRR-core, ERE, GARE-motif, P-box, TATC-box, TGA-element), circadian elements, and stress-responsive elements (ARE, DRE, LTR, MBS, TC-rich repeats), which are crucial for the plant's adaptability to environmental challenges were unevenly distributed in the promoter regions of the *BjCKXs*. Besides this, the presence of the growth, and development-specific elements (WRE3 and WUN), and the elements associated with endosperm-specific expression (GCN4_motif) and regulations reflect the BjCKX role in plant growth and development. Additionally, elements related to cell differentiation and cell cycle regulation (MSA-like) were present in specific gene promoters (Fig. 5). These findings indicate the diverse role of *BjCKXs* genes based on their gene structure and cis-regulatory elements distribution in their promoters.

Chromosomal distribution and duplication events of *BjCKX* genes

The *BjCKXs* genes were unevenly distributed in all the chromosomes of the amphidiploid (AABB, 2n = 36) *B. juncea* genome. For example, chromosomes B08 had 3 *BjCKXs*; A02, A03, A07, A09, A10, B02, B03, and B07 had 2 BjCKXs each; A04, A05, B04, B05, and B06 had only 1 *BjCKXs*; and A01, A06, A08, and B01 had no *BjCKX* (Fig. 6). The expansion of a gene family usually occurs due to the duplication events arising in the whole genome

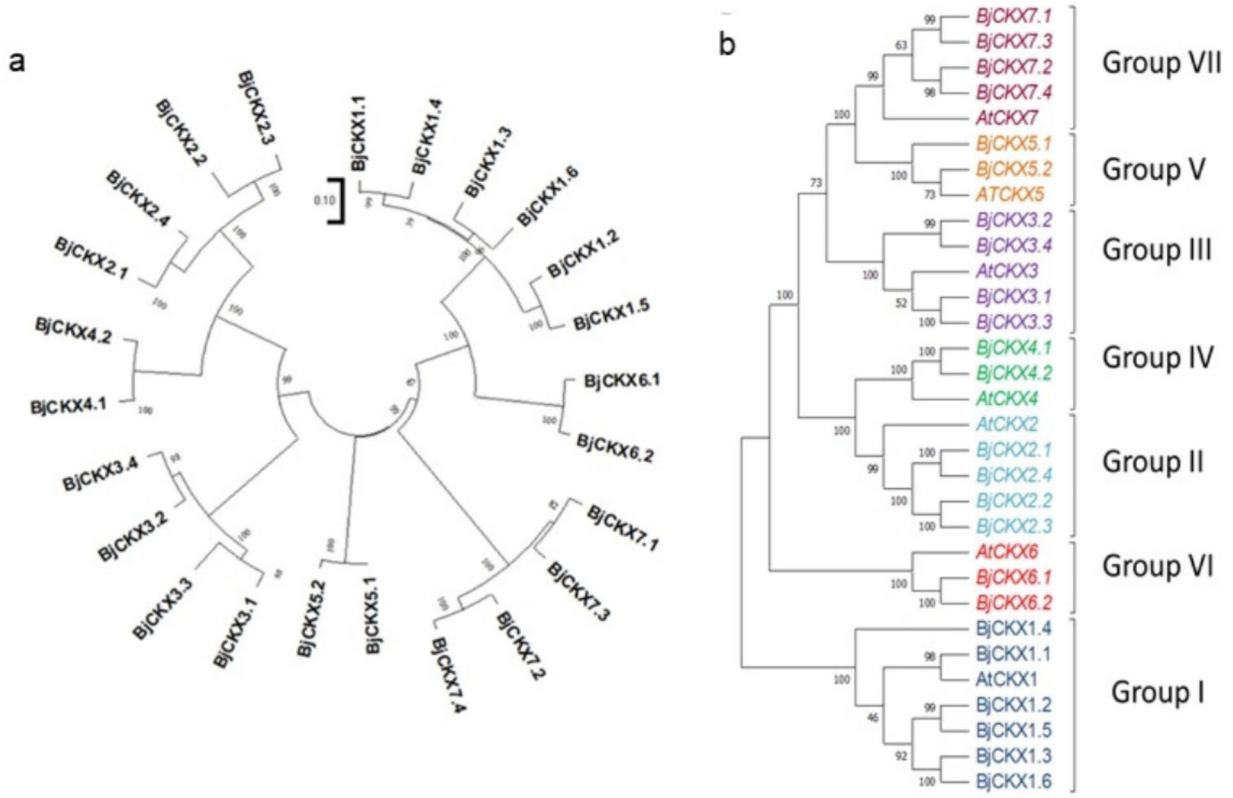

Fig. 2. The phylogenetic tree of *B. juncea* and *A. thaliana CKX* proteins. (**a**) The maximum likelihood phylogenetic tree was constructed using MEGA 11 with 1000 bootstrap replicates, (**b**) The phylogenetic tree was clustered into 7 subclades (I–VII). A distinct colour represents each subclade.

which was evident from the present study with the identification of the duplicate gene pairs among the *BjCKXs* present in the allopolyploid *B. juncea* genome (AABB) and their progenitor diploid species *B. rapa* (n = 10) with AA and *B. nigra* (n = 8) with BB genome. The A and B genomes in *B. juncea* were found to share equal numbers of BjCKX distribution, 12 on each A and B sets of chromosomes. The coding sequence comparison of 24 *BjCKX* genes has shown 17 *BjCKX* were collinear with the *B. rapa CKX* and 20 *BjCKX* with the *B. nigra CKX*. The collinearity of the *BjCKX* with their counterparts in *B. rapa* and *B. nigra* revealed that the gene family expansion was due to the segmental and tandem duplication of the chromosomal segments (Fig. 7). Further, the non-synonymous (K_A) and synonymous (K_s) substitution rate and the ratio (K_A/K_s) for the *BjCKX* gene family have shown the values of K_A vary from 0.0092 to 0.6107 and the K_S in the range of 0.1434 to 0.5053. The ratio of K_A/K_S was observed to be less than 1 (maximum = 0.323116) indicating a negative or purifying selection that tells that the duplicated genes were selected stably without losing their function. These findings demonstrated that the purifying or negative selection was involved in maintaining the conservation of the BjCKX gene structure during domestication or evolution. The syntenic relationships among different chromosomal segments of different species describe the origin of gene family members. The synteny analysis between CKX genes from the *B. rapa*, *B. nigra*, and *B. juncea* genomes (Fig. 7) revealed that 12 *BjCKXs* were placed in 12 colinear blocks with *B. rapa*, and 12 *BjCKXs* in 12 colinear blocks in *B. nigra*.

The comparative expression profiling of *BjCKX* genes in various tissues

For this, the two cultivars of *B. juncea,* RLM 198 and Pusa Jaikisan (PJK) which differ in the plant architecture and yield parameters were analyzed for the morphological features (Fig. 8) and the variation in the *CKX* gene expression in various tissues. The 100-day-old plants of the RLM 198 were observed taller (180.8 cm), thin (14.6 cm) and bearing fewer numbers of tertiary branches (6) compared to PJK with 175 cm of plant height, 16.56 cm of stem diameter, and 21 tertiary branches. Dimensions of the 3rd leaf from the ground in 50-day-old PJK plants had a larger leaf area (L: W-33 cm:16 cm) than RLM 198 (26 cm: 12.75 cm). The flower morphology in 60-day-old PJK plants had a broader and thicker gynoecium than RLM 198. The yield parameters in 100-day PJK plants had more numbers of siliques per plant (509) than RLM 198 (259). The number of siliques on the main raceme of the PJK was observed slightly more (52) than the RLM 198 (48). The average number of seeds per silique has been found more in PJK (14–18) than in RLM 198 (13–15). The seed size and thousand seed weight (test weight) were found more in PJK (5.58 mm and 5.99 g) than in RLM 198 (5.19 mm and 5.13 g). Overall, among the two cultivars, PJK was comparatively found superior to the RLM 198 in terms of the morphological features and yield characteristics as shown in Fig. 8.

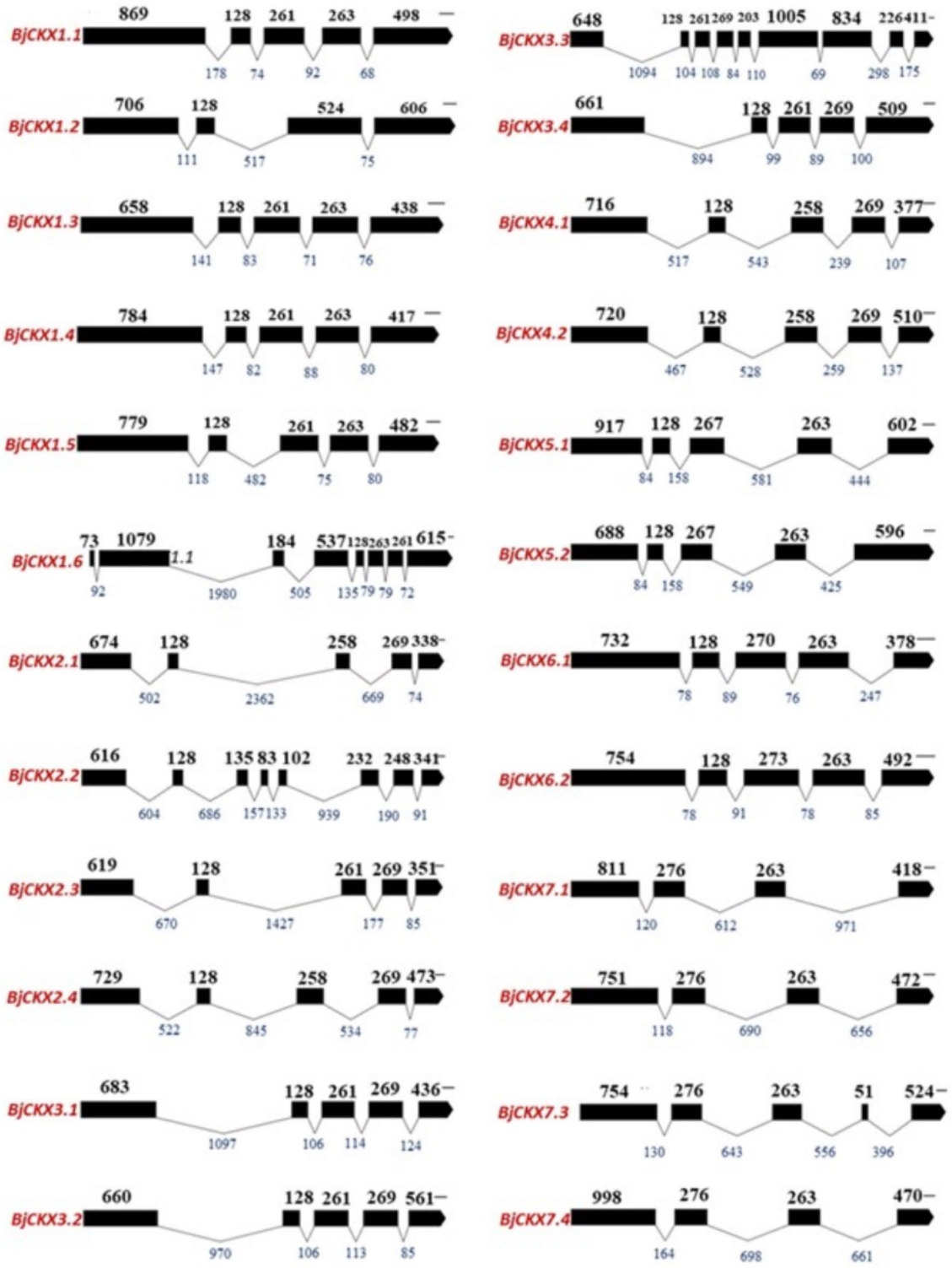

Fig. 3. Gene structure of CKX genes in *B. juncea*. Schematic representation of the exon–intron distribution in the *CKX* genes of *B. juncea*. The blocks represent exons and the line joining the blocks represents introns. Numerical values above the bars and below the thin lines are the size of the exons, and introns, respectively in base pairs. The bar above the last exon represents the 100 bp scale.

For the expression profiling of all 24 BjCKX, four different plant tissues, leaf, stem junction, flower buds and siliques were selected to reveal the CKX influencing the later three tissues. The assumption behind the selection of stem junction, flower buds and siliques was to recognize the CKX having their influence over the stem branching, floral primordia formation and the silique development. With RT-qPCR the expression pattern

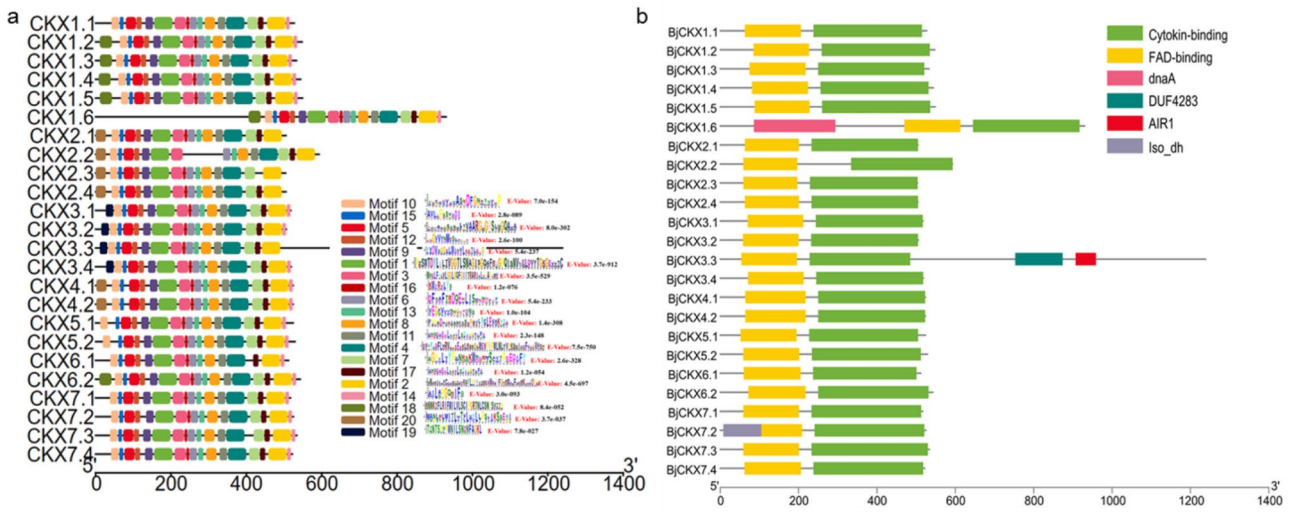

Fig. 4. Gene structure of CKX genes in *B. juncea*. (**a**) Conserved motif analysis was conducted using MEME Suite. A total of 20 motifs were predicted. (**b**) The domain organization of *BjCKXs*.

of all the *BjCKX* was observed to significantly change for some of them in two different cultivars represented in the heatmap (Fig. 9). For instance, in leaf tissues, *BjCKX1.1*, *1.3*, *1.4*, *1.5*, *1.6*, *2.4*, *4.1*, *5.1* and *7.1* exhibited higher expression in RLM 198 as compared to the *BjCKX1.2*, *2.2*, *2.3*, *3.1*, *3.2*, *3.3*, *3.4*, *6.1*, *6.2*, and *7.2* those expressions was high in PJK and the remaining *BjCKX2.1*, *2.2*, *4.2*, *5.2*, *7.3* and *7.4* had no significant difference in their expression in both the cultivars. In stem junction tissues, *BjCKX1.1*, *1.5*, *2.4*, *3.3, 6.1*, *6.2* and *7.4* expression was higher in the RLM compared to PJK, whereas the expression of the *BjCKX1.2*, *1.4*, *1.5*, *1.6*, *3.1*, *3.2*, *3.4*, and *4.2* genes was higher in PJK than RLM-198, and no significant differences were observed in the expression of the *BjCKX1.3*, *2.1*, *2.2*, *2.4*, *5.1*, *5.2*, *7.1*, *7.2* and *7.3* genes in both the cultivars. In flower bud tissues, the *BjCKX3.1, 3.4, 4.1, 4.2, 5.1, 5.2, 6.1, 6.2, 7.1, 7.3,* and *7.4* genes had higher expression in the RLM 198, whereas the B*jCKX1.1, 1.2, 1.3, 1.5, 1.6, 2.1, 2.2, 2.3*, and *2.4* genes were having the higher expression in PJK. However, no significant differences were observed in the expression of the *BjCKX1.4* and *7.2* genes in flower bud tissues in both cultivars. In 10- day old siliques, RLM 198 exhibited higher expression of the *BjCKX1.1*, *1.4*, *1.5*, *1.6*, *3.2*, *3.3*, *3.4*, *4.1*, *4.2*, *5.2*, *6.2*, *7.1* and *7.3* genes, whereas, expression of the *BjCKX1.3*, *2.1*, *2.2*, *2.3*, *2.4*, *3.1* and *5.1* genes were comparatively higher in PJK. However, no significant differences in expression were found for the *BjCKX1.2*, *5.2*, *7.2* and *7.4* genes in silique tissues.

Selection of the *BjCKX* for yield trait

The selection criteria for the CKX have been made on their expression pattern in the stem junction, flower buds and silique tissues of the two contrasting cultivars of *B. juncea*. The fold change in the expression level of the *BjCKX* in RLM 198 and PJK in four different tissues leaf (Fig. 10a), stem junction (Fig. 10b), flower buds (Fig. 10c), and in siliques (Fig. 10d) has shown the *BjCKX3.1*, *3.4*, *5.2* and *6.1* had the higher expression in the flower buds of the RLM 198 than PJK. In siliques, expression of the *BjCKX1.1*, *1.4*, *1.5*, *1.6*, *3.2, 3.4*, *4.1*, *4.2*, *6.2* and *7.3* were found higher in RLM 198 than PJK. Among these, the expression of the *BjCKX3.4* was comparatively higher in flower buds (~ 54 fold) and siliques (~ tenfold) of RLM 198 whereas, the expression of the *BjCKX5.2* was higher in the flower buds (~ 51 fold) of RLM 198 than PJK.

Furthermore, the phylogenetic analysis of all 24 *BjCKX* with functionally characterized yield-influencing *CKX* in other plant species including Arabidopsis, *B. napus*, Rice, Wheat, and Barley has shown the homology with *BjCKX3.4* and *BjCKX5.2* genes (Fig. 11a). The *BjCKX3.4* was observed closely associated with *AtCKX3*, *BnCKX3* A1 and *BnCKX3* C1, and they were grouped in the same clade, whereas the *BjCKX3.1* was grouped with *BnCKX3* A2 as shown in Fig. 11b. The closeness of *BjCKX5.2* was found with the yield-influencing *AtCKX5*, *BnCKX5.2*-A1 and *BnCKX5.2*-C1 and grouped in the same clad, whereas *BjCKX5.1* was observed closely associated with *TaCKX6*. The *BjCKX7.1* and *BjCKX7.4* have shared the phylogenetic similarity and grouped with *OsCKX11* of rice (Fig. 11c). Our results indicate that *BjCKX3.4* and *BjCKX5.2* might play a pivotal role in regulating the flower buds and silique length under normal growth conditions.

Discussion

The rapeseed/Indian mustard (*B. juncea*) is an allotetraploid crop with an AABB genome (2n = 36) and is an important source of edible oil especially in India. However, the productivity of the crop is comparatively lower than the other countries including China, Canada, and Australia. Cytokinin oxidase/dehydrogenase (EC.1.5.99.12) is an essential enzyme that irreversibly breaks down cytokinin by cleaving its side chain, rendering it inactive[2]. This enzyme plays a crucial role in regulating cytokinin homeostasis in plants and regulates the localized cytokinin concentration in various plant tissues. The genes for Cytokinin oxidase/dehydrogenase and related enzymes are found throughout various organisms, including bacteria, fungi, plants, and animals. Hence systematic studies

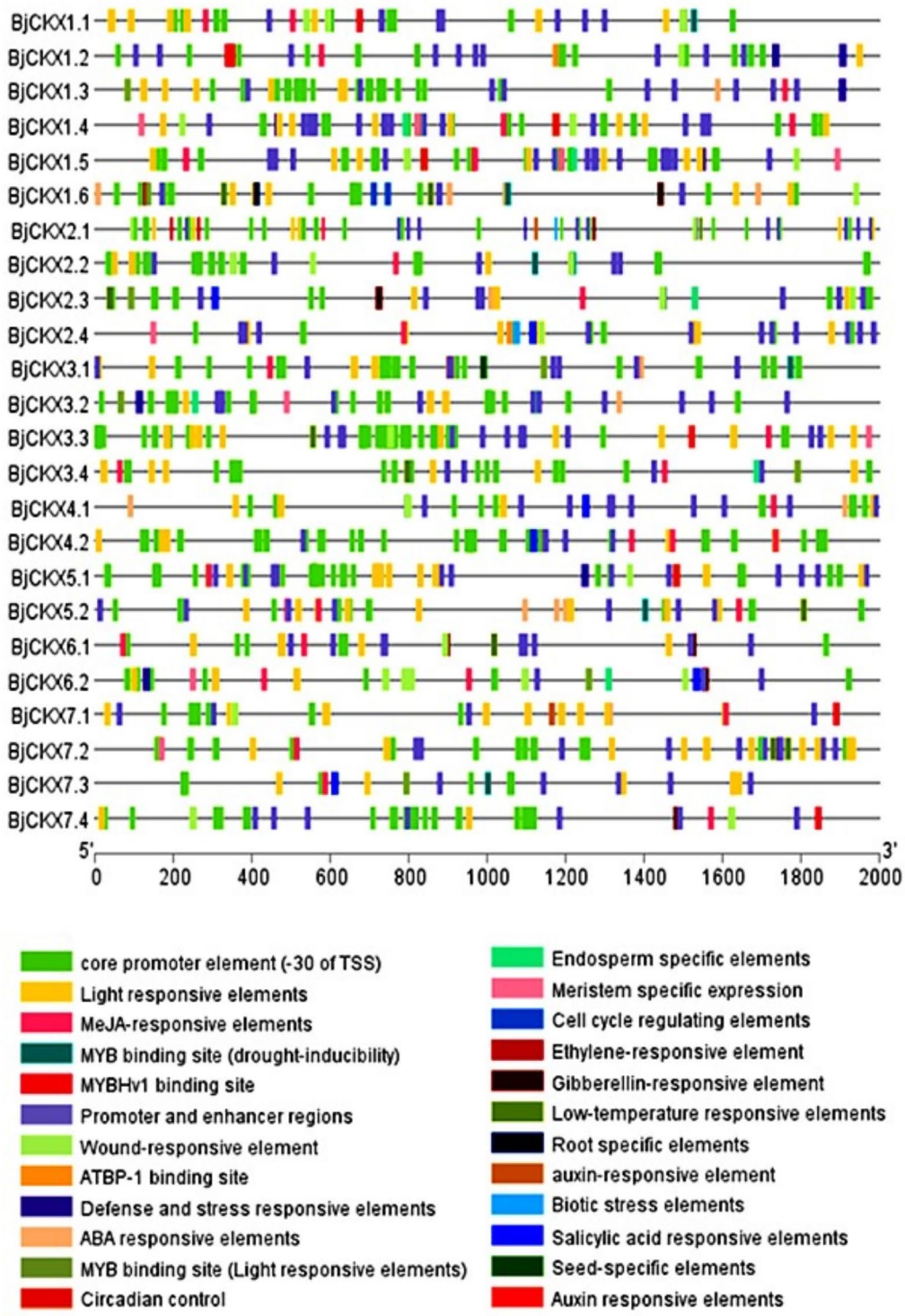

Fig. 5. The CRE analysis in the BjCKX promoter sequences. The 2 kb upstream regulatory sequences of the BjCKX gene promoter regions were extracted from the whole genome sequence of the *B. juncea* cv. Varuna and analyzed using the PlantCARE database.

of the CKX gene distribution throughout the genome and deciphering their biological functions are essentially required to know especially their role in the growth and development of the plants. The CKX gene family has been extensively studied in many monocots and dicot species such as *Oryza sativa*[18], *Triticum aestivum*[19], *Zea mays*[20], *Hordeum vulgare*[21], *Elusine corocana*[22], *Seteria italica*[23] and the dicots like *Arabidopsis thaliana*[24], *B. rapa,*[25], *B. oleracea,*[11], *B. napus*[26], *B. juncea*[27], *Glycine max*[29], *Cicer arietinum*[9], *Phaseolus vulgare*[9], *Medicago*

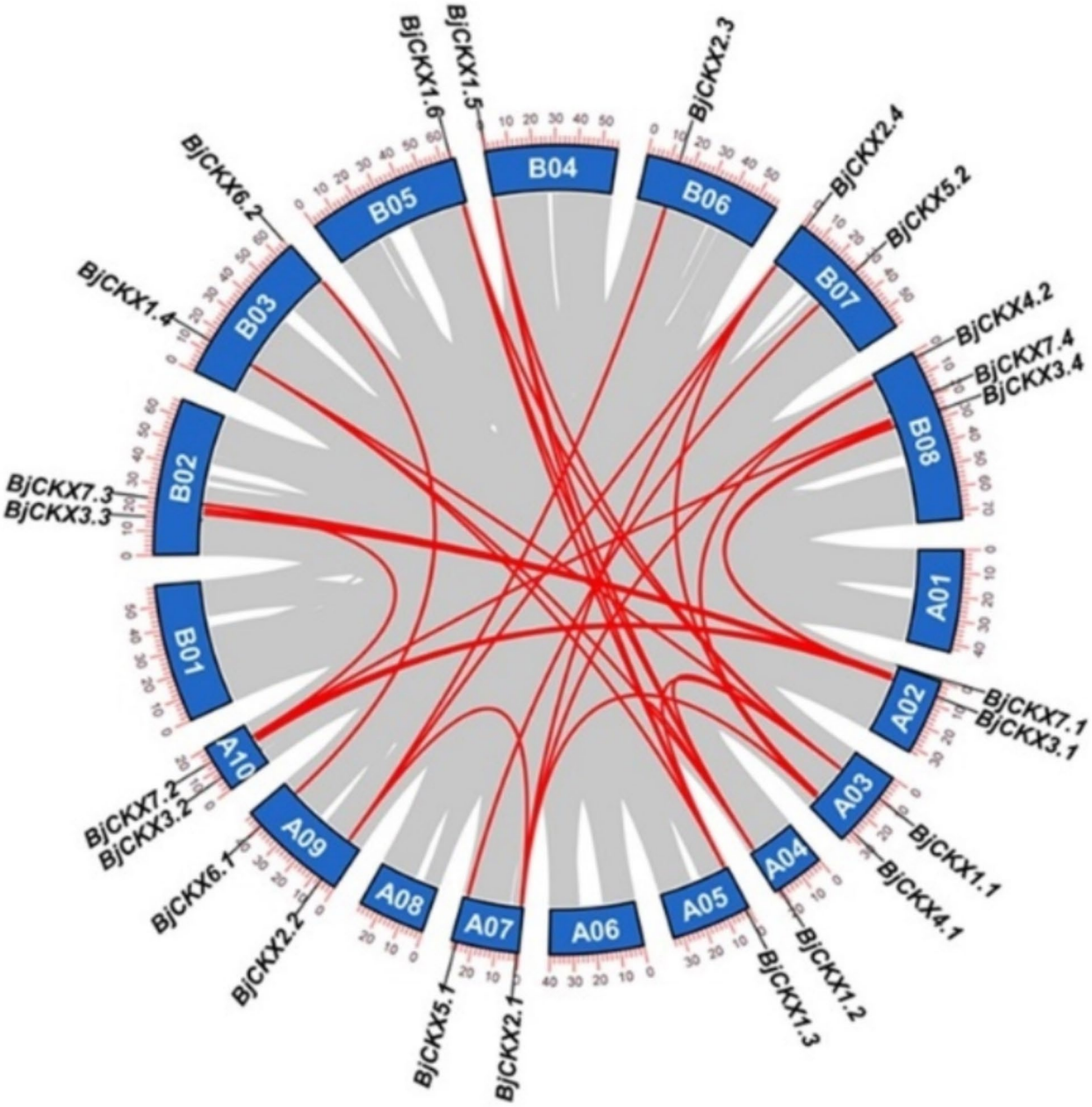

Fig. 6. Circos plot of CKX gene duplication in *B. juncea*. The different colours represent the genes found in different (I-VII subclades) subgroups, and the lines in the middle show segmental and tandem duplications between different chromosomes.

truncatula[30], *Jatropha curcas*[31], *Malus domestica*[32], while comprehensive identification of CKXs in *B. juncea* is still lacking.

In this study, we identified, a total of 24 *CKXs* genes in *B. juncea* cv. Varuna which is more than those reported in *B. rapa* (12)[25], *B. juncea* cv. Tumida (23)[27,28] and *B. napus* (23)[26], but less than those in *B. oleracea* (36)[11] and *T. aestivum* (35)[19]. This higher number of CKX genes in *B. juncea* might be owing to its allopolyploidy nature. We first analysed the physical location of the BjCKXs in *B. juncea* throughout its A and B genomes. We found that all the BjCKXs were unevenly distributed in all the sets of A and B chromosomes which is consistent with their chromosomal distribution in *B. rapa* (AA) (O'Keefe et al. 2011) and *B. nigra* (BB).

The earlier finding of the *BjCKX* gene family in the A and B genomes of the *B. juncea* cv. Tumida[27] and Ver2 chromosome Tumida[28] showed 23 BjCKXs as compared to our study where a total of 24 *BjCKXs* were identified in *B. juncea* cv. Varuna genome and thus the chromosomal distribution of them has also been observed to vary. The differences in numbers and their distribution might happen due to the variation in the quality and coverage of the *B. juncea* genome sequences. The phylogenetic annotation of these 24 BjCKXs placed them into 7 clades along with the *CKX* members of closely related crucifers. The distribution of BjCKXs on the 'A' set of *B. juncea*

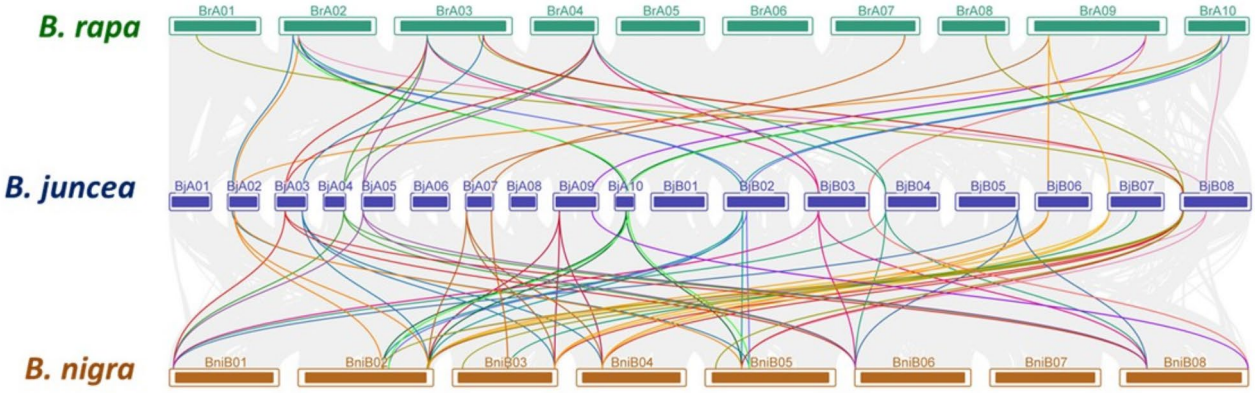

Fig. 7. Triple synteny plots between *B. rapa*, *B. juncea*, and *B. nigra* genomes, with orthologous CKX genes shown with connecting lines.

chromosomes was found in coherence with the distribution of the *BnCKX* and BrCKXs in the 'A' set of *B. napus* and *B. rapa* chromosomes, respectively[33].

Most of the predicted BjCKX proteins were observed in the range of 505 amino acids with some of them to 1239 amino acids. Two proteins BjCKX1.6 and BjCKX3.3 were observed with 930 and 1239 amino acid residues, respectively. BjCKX09 in *B. juncea*[27] and BnCKX2-1 in *B. napus*[26] have also been reported with the 1337 and 768, amino acid residues, respectively. The crystal structure of the CKX in Arabidopsis[34] and *Zea mays*[35] has shown that most of them have consisted of ~ 500 to ~ 600 amino acid residues. The isoelectric point (pI) of the predicted BjCKX was found in the range of 5 to 11. The *BjCKX* family members have shown similar gene structure and features including the motifs, domains, and exons-introns distribution. Several motifs were found conserved and exclusively present in all the *CKX* gene family members, whereas, a few other motifs were additionally found in some of the members. All the members were conserved with two functional domains namely FAD and Cytokinin binding domains. In addition to the typical conserved domains, BjCKX1.6 has dnaA (PRK14086) domains and BjCKX3.3 has two additional domains namely DUF4283 (cl16623) and AIR1 domains (COG5082) whose role was found in post-translational modifying proteins. Domains with a similar function were observed in BrCKX1.3 of *B. rapa*[25] and the *TPR15* gene adjacent to the *AtCKX1* in Arabidopsis[33] and both have a role in post-translational modification. These two genes were fused and found in the *BjCKX3.3* of the *Brassica* genome. However, probable assembly error leads to considering the two genes together in the CDS. Number of exons was found to vary from 4 to 9 in all the predicted *BjCKX*. Concerning the size of the exons, uniformity was observed in most of them across the *BjCKX* in Varuna for instance the second exon of all the *BjCKX*s was 128 bp except for the *BjCKX* of clad 7 (276 bp). The subcellular localization of all the *BjCKX* has revealed their coherence with the phylogenetically grouped members and the members of an individual group shared the most common cellular localization. The subcellular localization of *BjCKX* predicted by the Plant mPLoC online tool has shown they were localized in vacuoles, endoplasmic reticulum, and extracellular spaces which were found uniform across the individual members of clad 1 to 7. The vacuoles are usually found accumulating the N-glucosylated cytokinin, which catabolizes through vacuole-localized CKX proteins. In Arabidopsis, GFP fused AtCKX1 and AtCKX3 were found to be localized to ER (endoplasmic reticulum) and vacuoles[36]. Meanwhile, AtCKX7-GFP overexpression has revealed their localization in the cytosol[37]. The subcellular localization prediction in *B. napus* for *BnCKX* has shown their distribution additionally in mitochondria and chloroplast[26]. The substrate specificity of the various AtCKX found varied based on their function and subcellular environment[38]. Among the *ZmCKX* gene family, most of them were observed localized in the vacuoles[39]. The Arabidopsis histidine kinase (AHK), a cytokinin signal receptor protein present on the plasma membrane and ER was found to function normally at neutral pH and lose its activity in the acidic pH (5) which may be one of the probable reasons why the cytokinin signals being perceived mainly through ER-localized AHK protein[40]. The ER-localized *CKX* might have a role in the regulation of cytokinin in ER and affect signal perception. The effects of the pH on *CKX* gene function vary between the members depending on their localization corresponding to the extracellular (acidic) and intracellular environment (neutral). CKX usually has the optimum activity at both acidic and neutral pH suggesting their involvement in the regulation of cytokinin in both extracellular and intracellular levels[41,42].

Various *cis*-regulatory elements (CRE) in the upstream sequences (~ 2000 bp) of the predicted *BjCKXs* were found composed of mainly four elements, including core elements (TATA-box and CAAT-box), light-responsive elements, phytohormone-responsive elements (auxin-responsive, gibberellin responsive, abscisic acid-responsive, stress-responsive, including stress hormone salicylic acid and methyl jasmonate responsive, drought-responsive, and low-temperature responsive), as well as growth-specific elements (such as meristem-specific, seed-specific, endosperm specific, and cell cycle-specific). In the identified putative yield-responsive CKXs namely 3.4, and 5.2, the promoter regions were found carrying the important CREs for meristem-specific expression, cell cycle regulation, seed-specific elements, enhancer regions that may play significant roles in respective *BjCKX* expression. Similar observations about the distribution of CRE have been made in *B. juncea* cv.

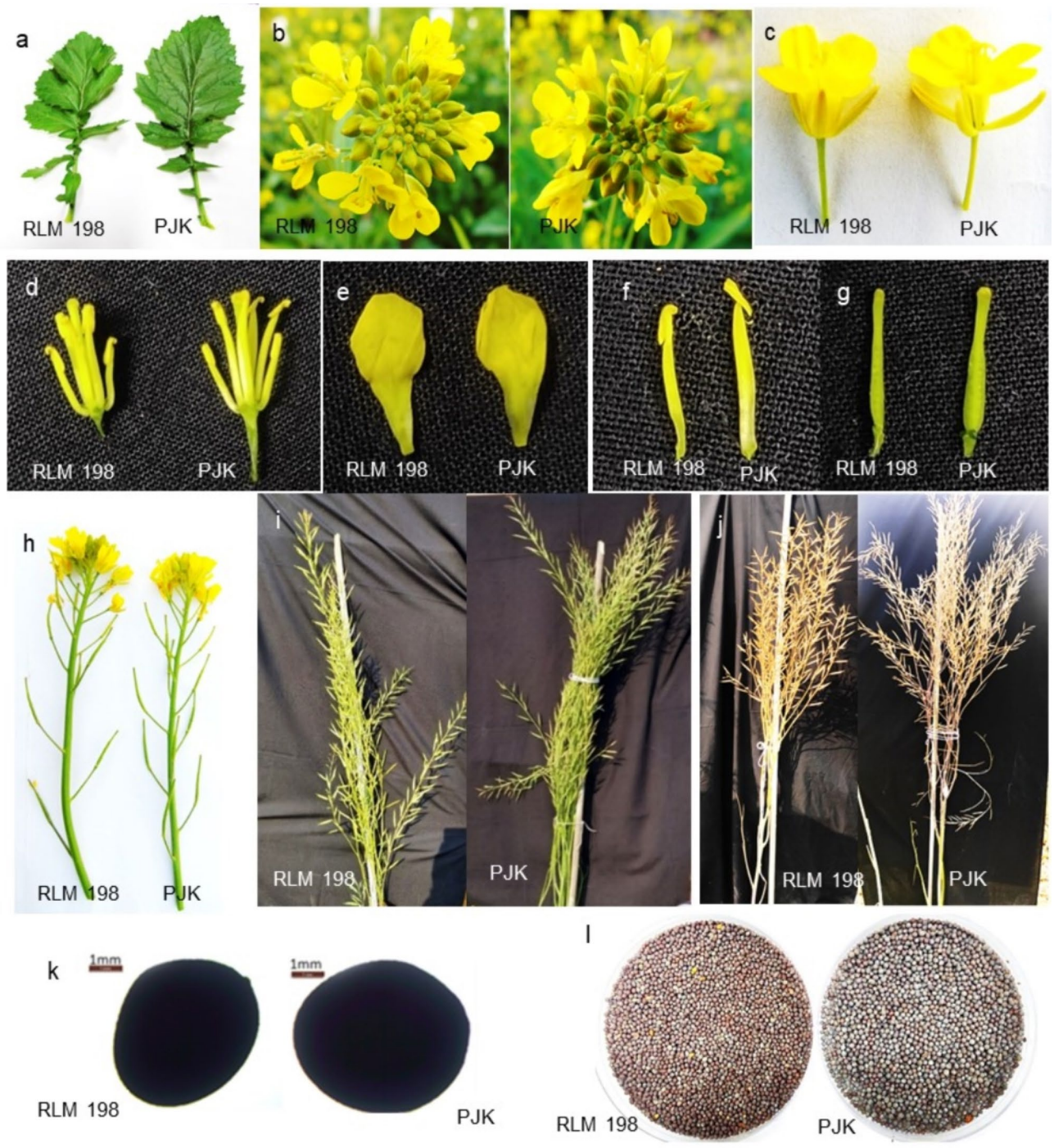

Fig. 8. Morphological features of the various plant tissues of RLM 198 and PJK genotypes of the *B. juncea*. (**a**) Leaf, (**b**) Flower buds, (**c**) Open flower (**d**) Petal (**e**) Reproductive part (**f**) Androecium (**g**) Gynoecium (**h**) Inflorescence (**i**) Siliques in plants at maturity (**j**) Siliques in plants at the harvesting stage (**k**) Individual mature seed (**l**) Seeds harvested after maturity.

Tumida[27], *B. rapa*[33], *B. napus*[26], and *B. oleracea*[11]. Interestingly, apart from *CKX*, the distribution of the CRE has also been found similar in the promoter regions of the other cytokinin metabolism genes like *IPT* (isopentenyl transferase) and *CGT* (Cytokinin Glucosyl transferase) in many crops like *B. rapa*[25], *B. napus*[43], and wheat[44].

The cytokinin has many roles in plant metabolism, and CKX plays a pivotal role in regulating the cytokinin concentration in different plant parts. The overexpression or downregulation of the specific CKX gene in developing seeds or in reproductive tissues has revealed its role in the seed's size, shape, and increase in their numbers[21,45]. The number of flower buds development is the crucial trait in determining the ovule numbers and seed per silique[46]. Hence, by analyzing the expression patterns of *BjCKXs* in four different tissues including

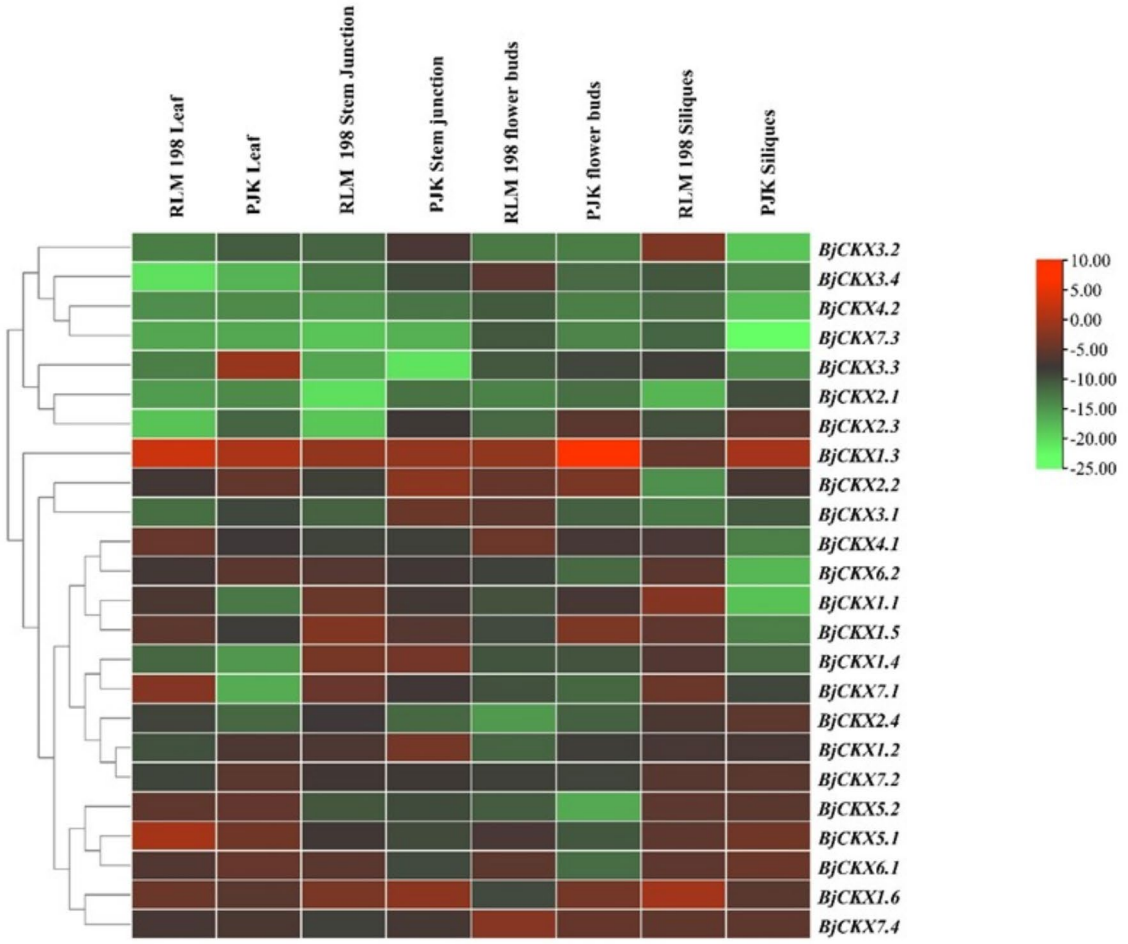

Fig. 9. Heatmap showing the differentially expressed *BjCKX* gene in leaf, stem junction, flower buds and silique tissues of RLM 198 and PJK genotypes of *B. juncea*. The colour in the heatmap from green to red represents the expression values from low to high. The individual box shows the expression values of a particular *BjCKX* in the respective tissue of the RLM 198 and PJK genotypes.

unopened flower buds, siliques, stem junction, and leaf tissues in high (PJK) and low-yielding (RLM 198) *B. juncea* cultivars showed large variations in their expression profile. These observations underscore the various roles of the *BjCKX* in different tissues and their potential influence over several traits.

Individual CKX genes are usually sensitive for a few traits whereas multiple CKX may have a larger effect over the number of traits. In shorting out the yield-responsive BjCKXs, we found that *BjCKX 3.1*, *3.4*, *5.2*, and *6.1* genes showed higher expression (> 30 fold) in flower buds of RLM 198 than PJK, which indicates the cytokinin level will be more in the PJK flower buds and that could be one of the reasons of having a larger number of flower buds in it (Fig. 8). In previous studies, the expression profile of *BnCKX3* and *BnCKX5* in *B. napus* was found more in flower as compared to the other BnCKXs, and the knockout of *BnCKX3* and *BnCKX5* together have shown increased numbers of flower buds and seed weight[17]. In rice, the knockout of inflorescence meristem-specific *OsCKX2* showed an increased grain number[47]. The phylogenetic association of *BjCKX3.4* with *BnCKX3-A1*, *BnCKX* 3-*C1*, and *AtCKX3* genes and *BjCKX5.2* with *BnCKX5-A1, BnCKX5-C1,* and *AtCKX3* led the way to analyze the *BjCKX3.4* and *BjCKX5*.2 expression pattern in two contrasting cultivars of *B. juncea*. The knockout of the *OsCKX11* of rice along with *OsCKX8* resulted in the increased grain number in the rice panicle[47], and their phylogenetic associations were found with *BjCKX7.1* and *BjCKX7.4*. Similarly, *BjCKX5.1*, *BjCKX7.2*, *BjCKX7.3*, and *BjCKX7.4* were related to distantly related monocots rice and wheat, showing less expression difference between RLM 198 and PJK compared to *BjCKX3.4* and *BjCKX5.2* in the flower buds. Based on the spatial expression differences and phylogenetic relationships with yield-influencing *CKX* from other dicot species, *BjCKX3.4* and *BjCKX5.2* were putatively selected for yield-governing candidate genes in *B. juncea*. Further analyzing the gene sequence information of *BjCKX3.4* and *BjCKX5.2* from RLM 198 and PJK showed no variation in the *BjCKX3.4* of the two cultivars. Meanwhile, the fourth intron of *BjCKX5.2* contains base pair substitution of cytosine to thymine at two regions + 2291 and + 2355 in PJK. There are several pieces of evidence showing that mutation in the intron regions affects the traits of the plants. In watermelon, a mutation in the first intron of gibberellin 3β-hydroxylase (*GA3ox*) affects the alternate splicing, thereby resulting in two isoforms ultimately having dwarf plants[48]. These results provide useful insights into the list of the *BjCKX* genes

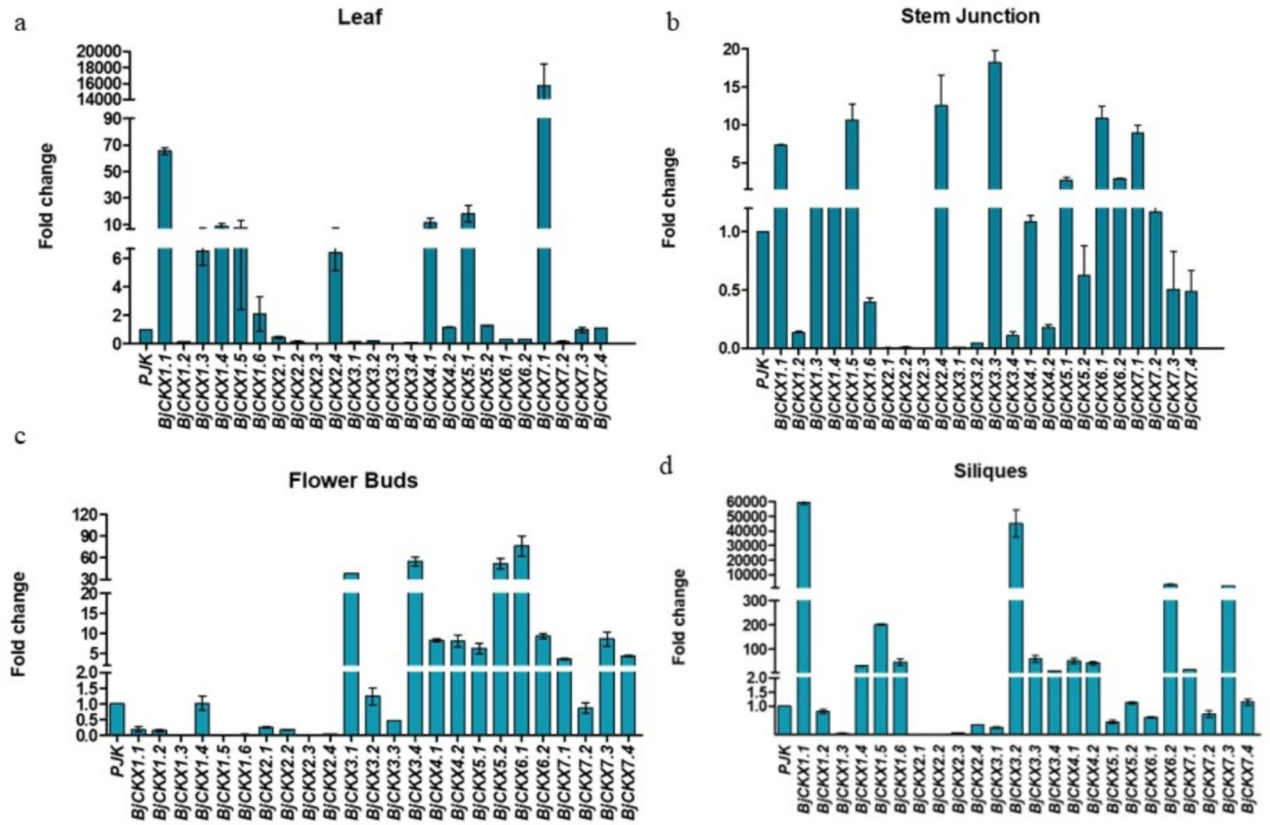

Fig. 10. The bar diagram shows the fold change in the expression values of the *BjCKX* genes in different tissues of the RLM 198 and PJK genotypes. (**a**) Leaf, (**b**) Stem junction, (**c**) Flower buds and (**d**) silique tissues.

that would be potentially involved in yield determination in rapeseed-mustard which can be validated through editing the selected genes.

Conclusion

Deciphering the CKX gene family member in the whole genome sequences of *B. juncea* cv. Varuna has led to the identification of 24 BjCKXs which were annotated in 7 groups and subgroups based on their sequence homology and phylogenetic association with their counterparts present in Arabidopsis and *B. napus*. With the comprehensive evolutionary relationship, expression profiles in different tissues and genetic analyses, we systematically revealed the structural features of all the *BjCKX* genes and their upstream promoter sequences, chromosomal distribution on the A and B genomes, synteny with the progenitor species namely *B. rapa,* and *B. nigra* of *B. juncea.* Two putative *BjCKX* homologs of the functionally validated yield-responsive CKXs in *A. thaliana* and *B. napus* have been identified in *B. juncea* which showed their relative expression higher in the unopened flower buds and silique tissues. Further functional validation of the predicted yield-responsive *BjCKX3.4* and *5.2* genes using knockdown (RNAi) or knockout (CRISPR/Cas9) is required to be done in rapeseed-mustard that may facilitate manipulation of the CKX gene family members in related oilseed *Brassica* species for crop improvement.

Methods

Mining of Bj*CKX* family in *B. juncea*

CKX gene sequence of *B. juncea* cv. Tumida obtained from the *Brassica* database (BRAD) and *(A) thaliana* from the TAIR database[49] was used as a query sequence in *(B) juncea* cv. Varuna whole-genome sequence using the BLASTn program of NCBI (https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/)[50]. The predicted *BjCKX* with an e-value < 0.001 were selected for further analysis. The coding sequence (CDS) of all the predicted CKX genes in *B. juncea* cv. Varuna were obtained through local blasts of their CDS datasets. The CDS and protein sequence of all the *BjCKX* genes of *B. juncea* cv. Varuna was analysed for the presence of the conserved domains, and motifs, respectively. The final CKX proteins were renamed according to their physical position on the *B. juncea* chromosome and their phylogenetic distribution along with their counterparts present in *A. thaliana*.

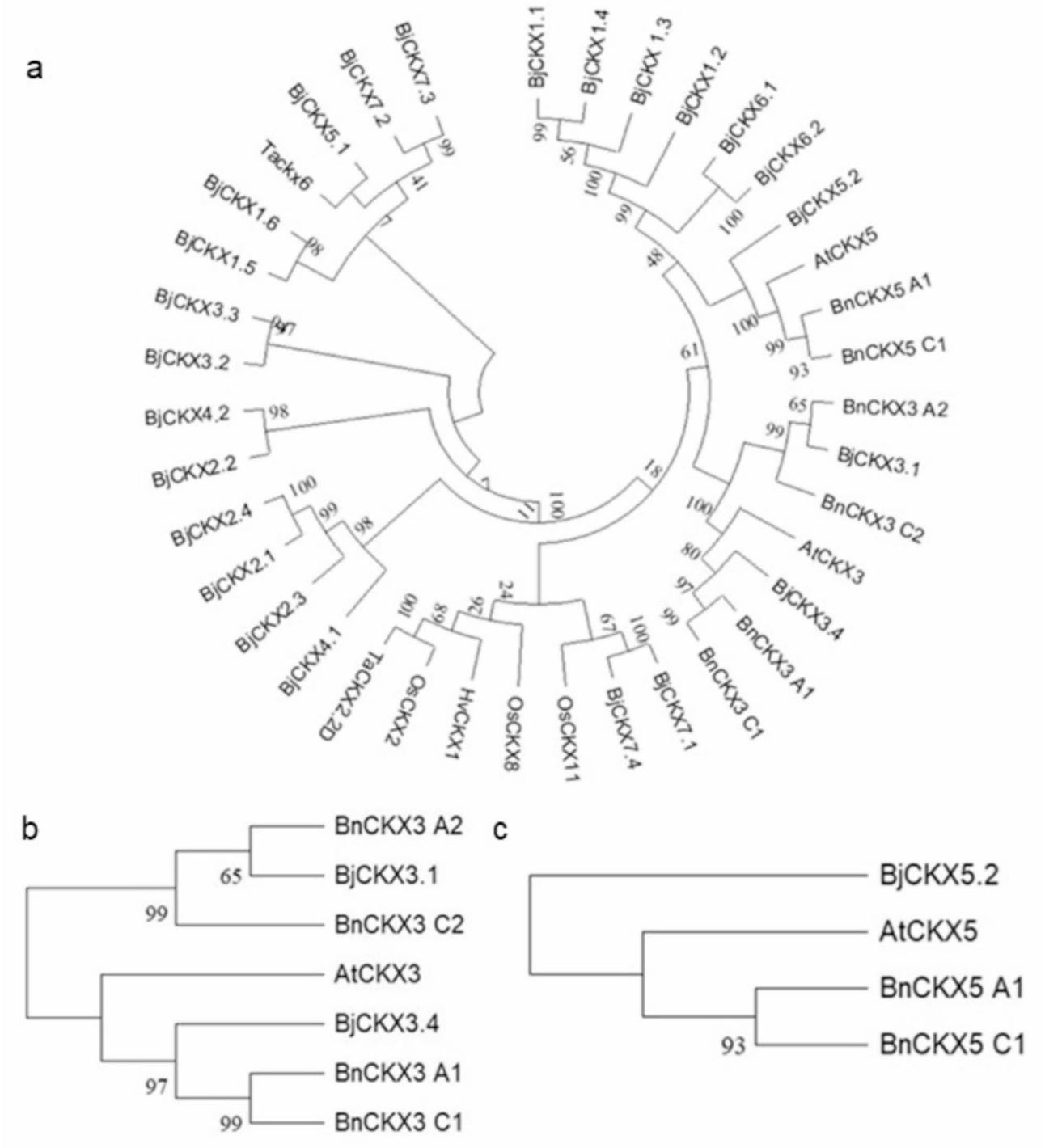

Fig. 11. The phylogenetic analysis of the *BjCKX* gene family with the functionally characterized yield influencing CKX of other plant species, (**a**) *BjCKX* association with known yield-responsive *CKX* genes (**b**) Subtree of *BjCKX3.4* with other yield influencing *CKX3* (**c**) Subtree of *BjCKX5.2* with other yield influencing *CKX5*.

Phylogeny of *B. Juncea* CKX proteins

For the phylogenetic analyses, the protein sequences of the *B. juncea* CKX were aligned with the *A. thaliana* CKX proteins by using the CLUSTALW and the Neighbor-joining tree was then generated using the MEGA 11 software[51] (https://www.megasoftware.net/). The bootstrap values were set at 1000 replicates.

Gene structural analysis of *B. Juncea* CKX proteins

The organizations of the intron and exon within the *BjCKX* genes were predicted using TBtools 2.003 (https://github.com/CJ-Chen/TBtools)[52]. The motifs were identified in the CDS sequences of all the predicted BjCKX by using the MEME suite (https://meme-suite.org/meme/tools/meme)[53] with the number of motifs set at 20. The domains were identified in their protein sequences by using the online tool CDD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)[54]. For the identification of the cis-regulatory elements within the promoters of the *BjCKX* genes, a 2 kb sequence upstream from the start codons was submitted to the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[55]. The physiochemical properties including protein length, molecular weight, and isoelectric point (PI*)* were predicted using the ProtParam tool (https://web.expasy.org/protparam/)[56], and subcellular localization using Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/)[57].

Gene duplication and evolutionary analysis of *BjCKX*

The duplication events and evolution studies of the BjCKXs family genes analysis were carried out using the MCScanX Wrapper program (https://github.com/wyp1125/MCScanX), of the TB tools (https://github.com/CJ-Chen/TBtools)[53] and visualized them in Circos. Furthermore, the Ka and Ks substitutions between the gene pairs were also calculated. Moreover, the syntenic relationship among *B. nigra*, *B. rapa*, and *B. juncea* was assessed using MCScanX and visualized in TB tools.

Plant material and yield attributing traits:

The timely sown field-grown plants of the *B. juncea* cultivars namely Pusa Jaikisan (PJK) and RLM 198, (cropping season 2022–23) were used for morphological and tissue-specific *BjCKX* gene expression studies. A panel of three biological replicates, each consisting of three plants, was used to measure the morphological datasets. No significant differences were observed among the replicates for each genotype. The selected genotypes were the pure lines of *B. juncea* cultivars Pusa Jaikisan and RLM 198 with significant differences in yield attributing traits. These two genotypes differ in seed size and overall seed yield. The morphological parameters of the leaves, flowers, and floral organs (calyx, corolla, androecium, and gynoecium) were measured in plants 60 days after sowing (DAS). The height of the plant, the diameter of the stem, the number of branches, siliques, seeds per silique, and seed weight were measured at the crop maturity stage i.e.,100 DAS. For the *BjCKX* expression studies leaf, stem junction, unopened flower buds of 68 DAS and siliques (10 days after anthesis) tissues were taken from PJK and RLM198 cultivars of *B. juncea* in three independent biological replicates. The samples were collected from similar plants at the same stage of both cultivars and immediately frozen in liquid nitrogen and stored at -80°C for subsequent use.

DNA isolation, primer synthesis and PCR analysis

The DNA from the leaf samples of the Pusa Jaikisan (PJK) and RLM198 was isolated by using the standard CTAB method and purified with RNaseA treatment. The *BjCKX*-specific primers were designed with unique base pairs at 3'end for all the predicted CKX in *B. juncea* cv. Varuna. These gene-specific primers were tested by PCR amplification in both the cultivars RLM 198 and PJK.

RNA isolation and real-time quantitative PCR expression analysis

The total RNA was extracted from the collected samples, by using the TRIzol reagent (Thermo Fischer Scientific, USA) following the standard method. The RNA concentration was measured by NanoDrop (Thermo Fischer Scientific, USA). DNase I treatment was given to all the RNA samples by following the manufacturer's protocol (Thermo Fischer Scientific, USA). The DNA-free RNA samples (2-5 µg) were checked with normal PCR with Taq DNA polymerase. The first complementary strand of DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, USA). The synthesized cDNA was diluted to 1:10 for qRT-PCR analysis. The cDNA of samples was assessed by RT-qPCR using TB Green® Premix Ex Taq™ II (Takara Bio, USA) for the reaction volume of 10 µl. The expression level of the Elongation factor-1α (EF-1α) of *B. juncea* was used as an internal control. The RT-qPCR was performed with three independent biological replicates with their three technical replicates for each sample using the Applied Biosystems Step One Real-Time Thermal cycler. The comparative expression profiling of all the *BjCKXs* in RLM 198 and PJK, 40- ΔcT was calculated and a graph was plotted[58]**.** The fold change was calculated for RLM 198 *BjCKX* with PJK as control. The ΔΔcT of RLM 198 were calculated by subtracting ΔcT RLM 198 from the ΔcT of PJK *BjCKX*. The relative expression level of *BjCKXs* was calculated using the $2^{-\Delta\Delta CT}$ method[59].

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request. The CKX datasets generated during and/or analysed during the current study are available in the Gene Bank repository with accession numbers BankIt2784537 BjCKX-1 to BjCKX-24 as PP102224 to PP102247, respectively (https://www.ncbi.nlm.nih.gov/genbank/update.html).

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Author contributions

All authors have read and approved the final manuscript. NCG has conceptualized, and brought the project grant. N.C.G., B.B. and K.G. worked on methodology. B.B. has performed all the wet lab experimentation and collected the plant datasets from the Brassica field. M.S., D.C. M., S.S., G.M., and M.R. were involved in bioinformatic analysis and data compilation. B.B. wrote the original draft of the manuscript. G.M., S.S., and MR have given critical inputs in finalising the manuscript. N.C.G. & K.G. reviewed, edited and finalized the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Guidelines statement

Experiments conducted in this study have been carried out following relevant institutional, national, and international guidelines and legislation.

Permissions/licences for the use of plant materials

The authors declare that they have obtained due permission for the collection and use of the *B. juncea* cultivars (Pusa Jaikisan/PJK and RLM 198) for the current study at the ICAR-NIPB, New Delhi.

Additional information

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