BMC Genomics

RESEARCH Open Access

Genome wide identification and characterization of *Bax inhibitor-1* gene family in cucumber (*Cucumis sativus*) under biotic and abiotic stress

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Abstract

In plants, the *BAX inhibitor-1* (*BI-1*) gene plays a crucial part in controlling cell death under stress conditions. This mechanism of Programmed Cell Death (PCD) is genetically regulated and is crucial for the elimination of unwanted or damaged cells in a controlled manner, which is essential for normal development and tissue maintenance. A study on cucumber identified and characterized five *BI-1* genes: *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4*, *and CsBI5*. These genes share conserved domains, indicating common evolutionary history and function. Physicochemical analysis revealed their molecular weights and isoelectric points, while subcellular localization showed their presence in different cellular compartments. The phylogenetic analysis highlighted evolutionary relationships with related crops. Chromosomal distribution and synteny analysis suggested segmental or tandem duplications within the gene family. Protein-protein interaction analysis revealed extensive interactions with other cucumber proteins. Cis-regulatory elements in the promoter regions provided insights into potential functions and transcriptional regulation. miRNAs showed diverse regulatory mechanisms, including mRNA cleavage and translational inhibition. The *CsBI3*, *CsBI4* and *CsBI5* genes exhibit elevated expression levels during cold stress, suggesting their vital involvement in cucumber plant defense mechanisms. The application of chitosan oligosaccharides externally confirms their distinct expression patterns. The qRT-PCR confirms the upregulation of *CsBI* genes in ToLCNDV-infected plants, indicating their potential to mitigate biotic and abiotic stresses. The comprehensive genome-wide exploration provides opportunities for the development of cold-tolerant and virus-resistant cucumber variants by traditional breeding or gene.

Keywords *Cucumis sativus*, *BAX inhibitor-1*, ER stress, Ca^{+2} level, Bioinformatics, Biotic stress, Abiotic stress

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Introduction

The *BAX Inhibitor-1 (BI-1)* gene family plays a pivotal role in plant resilience to both abiotic and biotic stresses. During adverse environmental conditions like drought, salinity, or pathogen attacks, *BI-1* genes are upregulated, regulating programmed cell death pathways to maintain cellular stability [1–4]. By inhibiting cell death, BI-1 proteins enhance stress tolerance and bolster plant defenses against pathogens, making them crucial regulators in plant stress responses [5]. Cucumber cultivation faces numerous biotic and abiotic challenges that result in substantial yield losses. About 80–97% yield losses were attributed to biotic and abiotic stress in cucumber [6, 7]. The *BI-1* gene family in cucumber reduces yield losses by enhancing stress tolerance and regulating defense mechanisms. It modulates cell death pathways during both biotic and abiotic stress, reducing pathogen spread and tissue damage, and ultimately improving plant health and yield [2, 3].

BAX inhibitor-1 (*BI-1*) is a eukaryotic conserved sensor localized in the endoplasmic reticulum (ER). BI-1 is a protein that was initially discovered by screening a complementary DNA library of humans in yeast [8]. BI-1 is commonly referred to as a "cell death suppressor" as its role in mammals involves preventing BAX-induced cell death by monitoring fluctuations in Ca^{+2} within the ER [9]. BI-1 has been found involved in anti-programmed cell death activity. This mechanism Programmed Cell Death (PCD) is genetically regulated and is crucial for the elimination of unwanted or damaged cells in a controlled manner which is essential for normal development and tissue maintenance [10]. BI-1 prevents autophagy in combination with cytochrome P450 and IRE1α in mammals [11]. BI-1 is evident to overcome PCD in plants induced by different stimuli [12, 13]. It is currently unclear if there is a direct interaction between BI-1 and IRE1 that regulate the pathways involved in PCD. *AtBI-1* is an additional sensor of eukaryotic ER stress that can detect changes in ER calcium stores and manage PCD [12]. *Arabidopsis thaliana BI-1* homologs in plants contribute to defense against externally introduced Bax, thus validating the capability of protective property of BI-1 homologs [14–16].

Previous research indicates that plant *BI-1* is linked to various environmental stress responses. For instance, overexpression of *BI-1* in Arabidopsis, tobacco, rice, cabbage, corn mildew and sugarcane enhances tolerance to oxidative, salt, and drought stresses [3, 17–23]. Conversely, BI-1 deletion increases sensitivity to heat shock and ER stress in plants. BI-1 also plays a role in resistance against pathogens like *Pseudomonas syringae* pv. tomato DC3000 carrying AvrRPT2, *Blumeria graminis* f. sp. tritici, *Botrytis cinerea*, *Chalara elegans*, *Puccinia striiformis*, and *Moniliophthora perniciosa* [24]. In wheat infected with *Fusarium graminearum*, the wheat *BI-1* gene (*Triticum aestivum* bax inhibitor-1) TaBI-1.1 was identified through RNA-sequence analysis [25, 26]. Understanding the *BI-1* mechanism could lead to the development of plants resilient to both abiotic and biotic stresses.

Cucumis sativus L. is among the earliest cultivated plants, and an important member of the Cucurbitaceae family [27]. Pakistan as an agrarian land, takes advantage of a suitable climate that allows year-round cultivation of vegetables. Cucumber is an annual vegetable crop having bisexual flowers, it can be a creeper or climber vine and completes its life cycle in 90–120 days [28]. It is believed that cucumber originated and bred in India while China is the largest producer with 75% annual production of the world [29]. Even so, over the past twenty years, viral diseases have significantly reduced the production of vegetable crops. Cucumber green mottle mosaic virus (CGMMV), Zucchini yellow mosaic virus (ZYMV), Watermelon mosaic virus (WMV) [30], Papaya ring spot virus (PRSV), Tomato leaf curl New Delhi virus (ToLCNDV), and Squash leaf curl China virus (SLCCNV) are among the viruses that cause yellow mosaic infection in members of Cucurbitaceae family [31]. Infections caused by Begomoviruses are responsible for the yellowing, folding, curling, cupping, etc. of leaves in cucumber. Many recent reports indicated a huge loss of current crops due to Begomoviruses infections which poses a serious thought to its remedy [32–35].

Cucumber is highly vulnerable to various diseases, leading to reduced yield and commercial losses. Cucumber can be encountered by both biotic and abiotic stresses [36]. Throughout its life cycle, cucumber in agriculture is subjected to unfavorable climatic circumstances such as salt, drought, and extreme temperatures, which have an unfavorable effect on crop productivity and growth [37]. Stress from both high and low temperatures dramatically modifies the molecular response [38]. Early diagnosis of diseases is needed to prevent the crop from severe infection [39]. To overcome these viral infections, various techniques were previously used and still new strategies are evolving like immunization [40, 41].

The primary goal of this study was to enhance our comprehension of genome-wide analysis and the role of the *BI-1* gene in conferring resistance against Begomoviruses. *BI-1*, which inhibits cell death, is present in both animals and plants [14, 42, 43]. The mRNA expression of *BI-1* has been studied in different tissues of plants which boost up during aging, biotic and abiotic stresses [44, 45]. During cold stress, *CsBI*1 and *CsBI*5 genes exhibit high expression levels, indicating their crucial role in the abiotic defense mechanism in cucumber plants. Cucumber plants infected with ToLCNDV showed increased expression levels of the *BI-1* genes as determined by qRT-PCR [46, 47], indicating their involvement in defending

against both biotic and abiotic stresses because of BI-1 involvement in crucial cellular processes and potential contribution to resistance against various biotic and abiotic stresses, *BI-1* presents a promising candidate for genome-wide analysis and comprehensive genomic research. Identifying and characterizing *BI-1* on a genomic scale provides opportunities for developing novel virus-resistant and cold-tolerant varieties through gene cloning or integration using conventional breeding approaches.

Materials and methods

Database search and sequence retrieval

To identify the *BI-1* gene in *C. sativus*, the protein sequence of the BAX1-I domain from *Arabidopsis thaliana* retrieved from NCBI (National Center for Biotechnology Information) was BLAST against the whole genome of *C. sativus* (Cucumber Chinese long v2), specifically the entry with the identifier PF01027. From the Motif Search [48] a sequence representing the BAX1-I domain was isolated. This particular sequence was utilized to search for *BI-1* in the Cucurbit Genomics Database (http://cucurbitgenomics.org/blast). The retrieved amino acid sequences were subjected to the Conserved Domain Database (CDD) at https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi using the default settings. All those proteins that did not contain the conserved domain (PF01027) corresponding to the BAX1-I domain were excluded from further analysis [49].

Determination of physio-chemical properties of cucumber (*Cucumis sativus*) BI-1 proteins

The amino acid length, molecular weight (Mw), and isoelectric point (pI) of the *BI-1* gene were estimated using the ProtParam tool [35, 50, 51]. Chromosomal position, chromosome direction, and CDS length size were all determined using the Cucurbit Genomics Database. Additionally, the WoLF PSORT online tool was used to predict the sub-cellular localization of *BI-1* [52].

Multiple sequence alignment and phylogenetic analysis

The *BI-1* gene's amino acid sequences were aligned using Clustal W 2.1, and the phylogeny was investigated using MEGA XI [48, 53]. The neighbor-joining method was employed with 1000 replications of bootstrapping and partial deletion.

Cis-regulatory elements, gene structure and conserved motifs recognition

The analysis of the promoter region involved obtaining a genomic sequence of 1000 upstream base pairs. The sequences were analyzed using the PlantCare database [33, 49] to predict *cis*-regulatory elements. To validate these predictions, the PLACE database (https://www.dna.affrc.go.jp/PLACE/) was consulted. Subsequently, the protein sequences of *BI-1* with the highest number of motifs were recognized by the MEME (Multiple EM for Motif Elicitation) program (https://meme-suite.org/meme/) [49]. To learn about the gene structure of BI-1 proteins, the Cucurbit Genomics Database was used to find their genomic and coding sequences. GSDS (Gene Structure Display Server) 2.0 was used to visualize the structure of the genes (http://gsds.gao-lab.org/) [33, 49].

Gene duplication analysis

The approximate divergence period of the cucumber (*Cucumis sativus*) *BI-1* gene family was calculated using the Ks and Ka values. The MUSCLE algorithm in the MEGA-X program was used to align the protein sequences [49]. Then, the Ka/Ks calculator from the TBTools software was used to determine the quantity of Ka and Ks substitution rates [54]. The Ka/Ks ratios were taken into account in order to estimate the rates of molecular evolution for each pair of paralogous genes [55–57].

Transcriptomic analysis

The Cucurbit Expression Atlas Cucurbit Genomics Database (CuGenDB) was used to locate the cucumber (*Cucumis sativus*) transcriptome data (PRJNA80169), and TBtools was used to do the tissue-specific expression analysis of 5 *CsBI* genes and displayed the results as a heatmap.

To examine the precise expression profile of *BI-1* in response to cold stress, the NCBI GEO database (Gene Expression Omnibus) was used. The GEO database was used to obtain RNA-sequence data of cucumber (*Cucumis sativus*) plants that had undergone treatment with chitosan oligosaccharide in the field (GSE224757) [58]. The Heatmap Illustrator tool in TBtools was then used to visualize the expression patterns and showed the links between the expression profiles using hierarchical clustering [59]. In addition, sequences taken from the GEO database were utilized to look at how they were expressed in response to the begomovirus [35].

Putative microRNA target sites analysis

The micro-RNA dataset for cucumber (*Cucumis sativus*) plants was downloaded from the Plant miRNA Encyclopedia (https://www.pmiren.com/download). By examining the coding sequence sequences, psRNA Target (https://plantgrn.noble.org/psRNATarget/analysis?function=3/) was used to search for miRNAs that target the *BI-1* genes of cucumber (*Cucumis sativus*) [55, 60].

Chromosomal location, synteny, and dual synteny analysis of *BI-1* genes

The Cucurbit Genomics Database was used to gather crucial data on the size of chromosomes and the precise placements of genes. Synteny analysis was carried out using the Advanced Circosprogramme in TBtools [61, 62]. The TBtools software's One Step MCScanX and Dual Synteny Plot were used to create gene con/uration similarity maps. These maps show how similar several species' gene configureurations are to one another [55, 63].

Analysis of protein-protein interaction network

By building protein interaction networks, the study examined the connections of all five cucumber (*Cucumis sativus*) proteins. This was done by using the Search tool in the STRING database v11.5, with various parameters and a default value of a medium confidence score of 0.400 [49, 64]. The STRING database version 11.5's local network clusters, KEGG pathways, biological processes, chemical activities, and other functional relationships were all present in the interaction network [55, 65].

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Physiological and bio-chemicals under virus stress

Chlorophyll content estimation

Fresh cucumber (*Cucumis sativus*) leaves were used for chlorophyll content estimation. Leaf extracts were dissolved in 80% acetone and the absorbance values for Chl. a, Chl. b, and total Chl [66]. were determined at 450 nm, 650 nm, and 663 nm using using a UV-Vis Spectrophotometer (AnalytikJena SPECORD-200) [67].

Peroxidase activity determination

To determine peroxidase activity, a 0.05 mol/L sodium phosphate buffer solution was prepared by diluting 0.2 mol/L buffer solution. A 0.3% H_2O_2 solution was prepared by diluting H_2O_2 with water, and a 0.2% Guaiacol solution was prepared by adding guaiacol to the buffer solution. Sample preparation involved mixing buffer solution, H_2O_2 solution, and plant extract, followed by absorbance measurement at 470 nm using a UV-Vis Spectrophotometer (AnalytikJena SPECORD-200) [68, 69].

Catalase activity determination

Catalase activity was quantified using a 0.05 mol/L sodium phosphate buffer solution and a 0.3% H_2O_2 solution. Sample preparation included mixing buffer solution, H_2O_2 solution, and plant extract, followed by absorbance measurement at 240 nm using a UV-Vis Spectrophotometer (AnalytikJena SPECORD-200) [66].

Superoxide dismutase (SOD) activity determination

SOD activity was quantified by adjusting the reaction mixture to a final volume of 3 mL with specific components. The mixture was incubated at room temperature, and the absorbance was measured at 560 nm using a UV-Vis Spectrophotometer (AnalytikJena SPECORD-200) [70, 71].

Effect of tomato leaf curl New Delhi virus (ToLCNDV) stress on cucumber (C. Sativus) varieties

Two cucumber (*Cucumis sativus*) varieties, "Oscar" and "ICSCU1652", were obtained and cultivated in a controlled environment at the Faculty of Agriculture Sciences, University of the Punjab, Lahore. A total of 300 seeds from both varieties were planted, and after 10–12 days, plants with uniform size and good health were selected for inoculation. Sixty plants from each variety were then divided into two groups named Cu-B1 and Cu-B2, with three replicates each. Inoculation with the ToLCNDV clone (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6460037/) was carried out using a syringe. Young leaf samples were collected from both control and inoculated plants when symptoms began to appear. These collected samples were promptly frozen in liquid nitrogen and stored at -80 °C. The remaining plants were harvested for subsequent analysis of their physical characteristics, physiological attributes, and biochemical components [72].

RNA extraction and qRT-PCR analysis

The plants were collected from the Departments of Horticulture, Punjab University, Lahore with voucher HDpu7598 and submitted to Horticultural Bank Punjab University Lahore. Young leaf tissues were used to isolate total RNA, and the Pure Link RNA Mini Kit from Invitrogen (Catalogue No. 12183018 A) was employed for this purpose. The RNA samples were quantified and standardized using NanoDrop Quantification. Primers for five *BI-1* genes were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Real-time expression analysis of the target genes having FPKM values were carried out using SYBR Select Master Mix (Catalogue No. 4472903), with cDNA as a template and the appropriate primers. Expression levels were quantified by calculating the $2^{-\Delta\Delta Ct}$ value relative to the control, and GAPDH was employed as an internal reference. The primer sets and protocols for quantifying virus titer were used as described [73].

Statistical analysis

The data was subjected to statistical analysis using a two-way factorial Randomized Complete Block Design (RCBD). Analysis of variance (ANOVA) was utilized to assess variances, and means were differentiated using the

least significant difference, with a significance level set at $P<0.05$. The entire statistical analysis was carried out using the Statistix 8.1 software.

Results

Identification of *BI-1* gene in *C. Sativus*

Six BI-1 proteins were identified. For the subsequent investigation, proteins resulting from identical gene isoforms or having truncated domains were ignored. Five different *BI-1* genes were eventually identified and used as the basis for further research.

Physio-chemical properties of *C. Sativus BI-1* gene

The *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4*, and *CsBI5* genes encode proteins that vary in length, typically between 238 and 293 amino acids. These proteins exhibit molecular weights ranging from 26.7 to 32.4 kD, with CsBI1 being the largest among them. The isoelectric points (pI) of these proteins range from 6.56 to 8.67, reflecting a diversity in their charge properties across different pH levels. This variation in amino acid length, molecular weight, and isoelectric points suggests that each protein might have distinct functional roles and stability under varying physiological conditions. The genes *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4*, and *CsBI5* are all localized in the plasma membrane. This specific localization is crucial as it plays a significant role in their functional dynamics within the cellular environment (Table 1).

Comparative phylogenetic analysis of *BI-1* proteins

The results showed four main clades emerging from the rooted tree, with a total of 81 BI-1 genes classified into Clade 1, Clade 2, Clade 3, and Clade 4. Clade 3 and Clade 4 were the largest groups, each consisting of twenty-three BI-1 genes. Clade 1 contained twenty-one BI-1 genes, while Clade 2 was the smallest group with fourteen BI-1 genes. These groups were formed based on the presence of Arabidopsis genes, indicating the evolutionary history of different crops. The functions of all genes in each clade may be similar to the Arabidopsis gene present in that clade (Fig. 1).

Sub-cellular localization signals in *BI-1* gene of *C. Sativus*

Sub-cellular location of *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4* and *CsBI5* genes was predicted in the plastid, endoplasmic reticulum, golgi apparatus and vacuole. *CsBI5* was highly localized in the plastid. *CsBI1* on the other hand, was typically located in the both plastid and golgi body. *CsBI5* amount was high in the plastid as compared to golgi body, ER and vacuole. The maximum localization of cucumber and tobacco BI-1 proteins was identified in the plasma membrane and vacuoles. In contrast, leeks showed high localization in the chloroplast (Fig. 2).

Analysis of conserved motif domains and gene structure in *C. Sativus BI-1* family genes

These non-redundant *BI-1* protein sequences from *C. sativus* exhibit highly conserved domains. All genes of *C. sativus* have conserved domain (Table 1S). Motif 1 was found present in all genes thus depicting it may have functional role in encoding *BI-1* protein. CsBI1, CsBI2, and CsBI3 genes contain the conserved motifs (8, 4, 12, 14, 6, 9, 20, 2, 1, and 10). CsBI4 and CsBI5 also have their own set of conserved motifs (1, 15, 19, 16, 17, 3, 18, 5, 13, 7, and 11). All five *BI-1* genes possess the BI-1 domain, which remains conserved across them (Table 2S). In silico analysis revealed that, number of exons varies from one to six in cucumber *BI-1*. *CsBI*, *CsBI2* and *CsBI3* each had 4 exons while *CsBI4* and *CsBI5* had 6 exons (Fig. 3a, b).

Analysis of protein-protein interaction network

The protein-protein interaction (PPI) analysis among BI-1 proteins revealed the complex networks of interactions.

Table 1 List of all five non-reduntant proteins observed in the genome of *C. Sativus*

Gene ID			Chr. No	Chromosome Location		Direction	Length Amino Acid		Isoelectric Point	Molecular weight (Mw)	Subcellular Localization
Rename	Cucumber (Chinese Long) v2	Cucumber (Chinese Long) v3		Start	End		Genomic	Peptide	(pI)	(KD)	
CsBI1	Csa6G061760.1	CsaV3_6G005470.1	6	4,664,965	4,667,387	F	735	244	6.56	27	Plasma Membrane
CsBI2	Csa2G222100.1	CsaV3_2G013450.1	2	10,735,003	10,736,757	F	717	238	7.74	26.7	Plasma Membrane
CsBI3	Csa2G223750.1	CsaV3_2G013630.1	2	10,894,380	10,896,518	R	726	241	8.62	26.9	Plasma Membrane
CsBI4	Csa3G912950.1	CsaV3_3G049850.1	3	39,597,173	39,599,440	R	882	293	7.12	32.4	Plasma Membrane
CsBI5	Csa3G912940.1	CsaV3_3G049840.1	3	39,593,461	39,595,587	R	753	250	8.67	27.6	Plasma Membrane

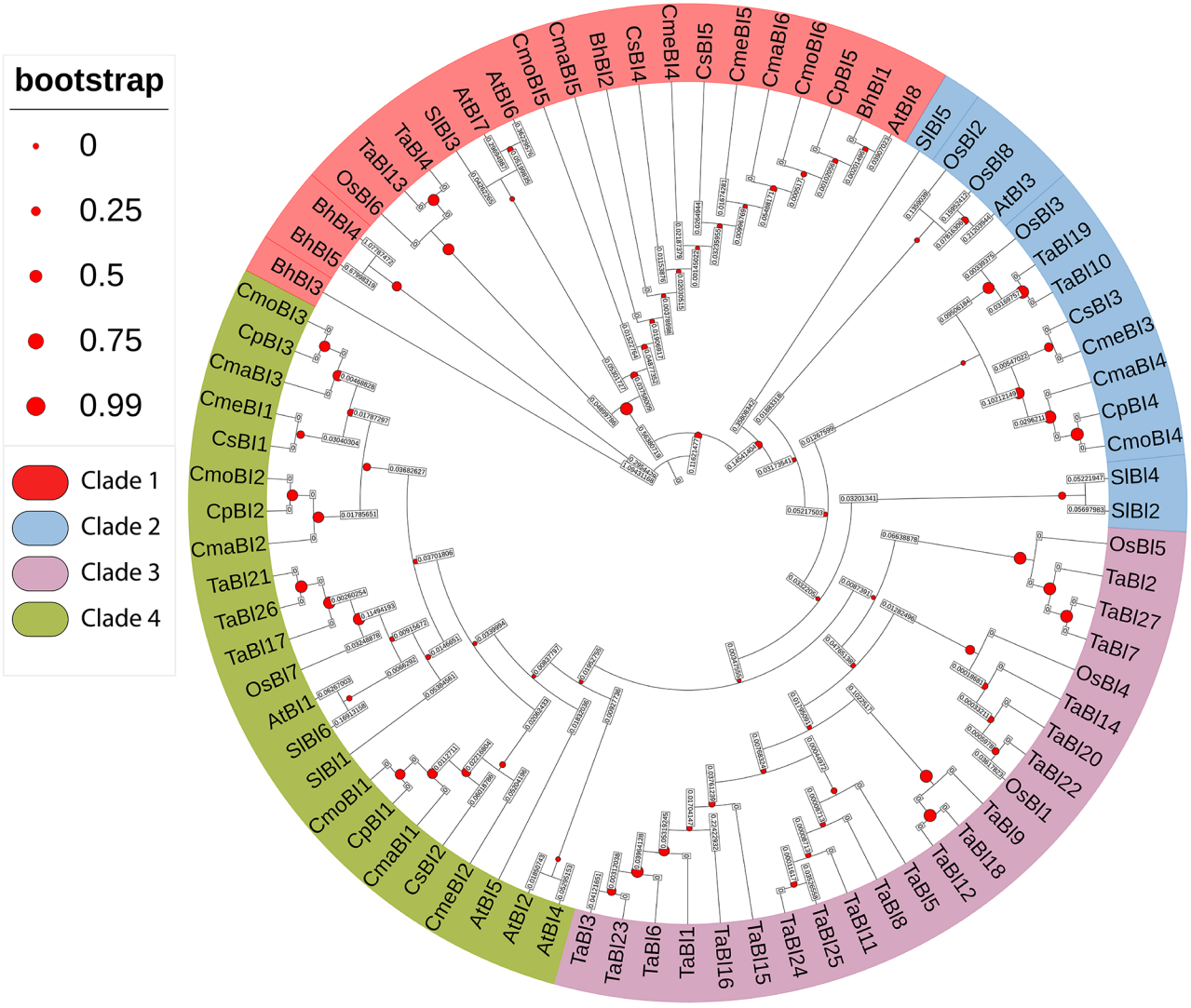

Fig. 1 The phylogenetic tree was generated based on the full-length sequences of BI-1 proteins from *C. sativus (Cs)*, *A. thaliana (At)*, *C. melo (Cm)*, *C. pepo (Cp)*, *C. moschata (Cm)*, *Oryza sativa (Os)*, *Triticum aestivum (Ts)*, *Benincasa hispida (Bh)*, *Solanum lycopersicum (Sl)*, *and C. maxima (Cm)*. Each clade was indicated with a specific color: Clade 1 (red), Clade 2 (blue), Clade 3 (purple), and Clade 4 (green)

With a minimum required interaction score indicating low confidence set at 0.150, the protein-protein interaction (PPI) network consisted of 5 nodes and 6 edges. The average node degree was 2.4, while the average low clustering coefficient reached 0, suggesting dense interconnectivity within the network. Notably, the expected number of edges was 0, underscoring significant enrichment of protein interactions. The PPI enrichment p-value was <1.29 e-12, emphasizing the substantial significance of the observed interactions.

BI-1 proteins CsBI1, CsBI2, CsBI3, CsBI4, and CsBI5 demonstrated interactions with themselves within the *C. sativus* genome (Fig. 4). This indicated that CsBI1 proteins were linked with CsBI4 and CsBI5, while CsBI2 proteins also exhibited linkages with CsBI4 and CsBI5. Similarly, CsBI3 proteins were connected with CsBI4 and CsBI5. These interactions suggested that these proteins might have had similar functions in cucumber (Fig. 4).

Synteny analysis and chromosomal location of *C. sativusBI-1* genes

During the synteny analysis both the genes i.e., *CsBI2* and *CsBI3* were present on chromosome 2 linked to *CsBI4* and *CsBI5* genes which were present on chromosome 3. The synteny analysis has revealed the presence of tandem duplication in *C. sativus*. Chromosomal distribution analysis of *C. sativus BI-1* genes demonstrated that *CsBI1* were located on chromosome 6, *CsBI2* and *CsBI3* on chromosome 2, and *CsBI4* and *CsBI5* on chromosome 3.

The dual synteny analysis has revealed the presence of segmented duplication. All of the *BI-1* genes in *C. sativus* have syntenic relationship with *BI-1* genes in all other

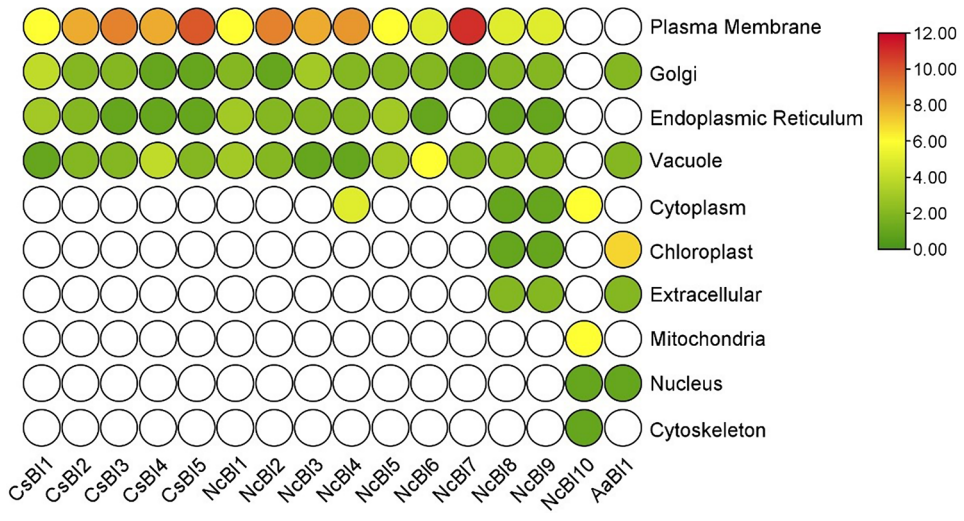

Fig. 2 Heat map showing the sub-cellular localization of *C. sativus* (*CsBI*), *Allium ampeloprasum* (AaBI) and *Nicotiana tabacum* (NtBI) proteins. Green color indicates the lowest and red color indicates the highest number of signals predicted

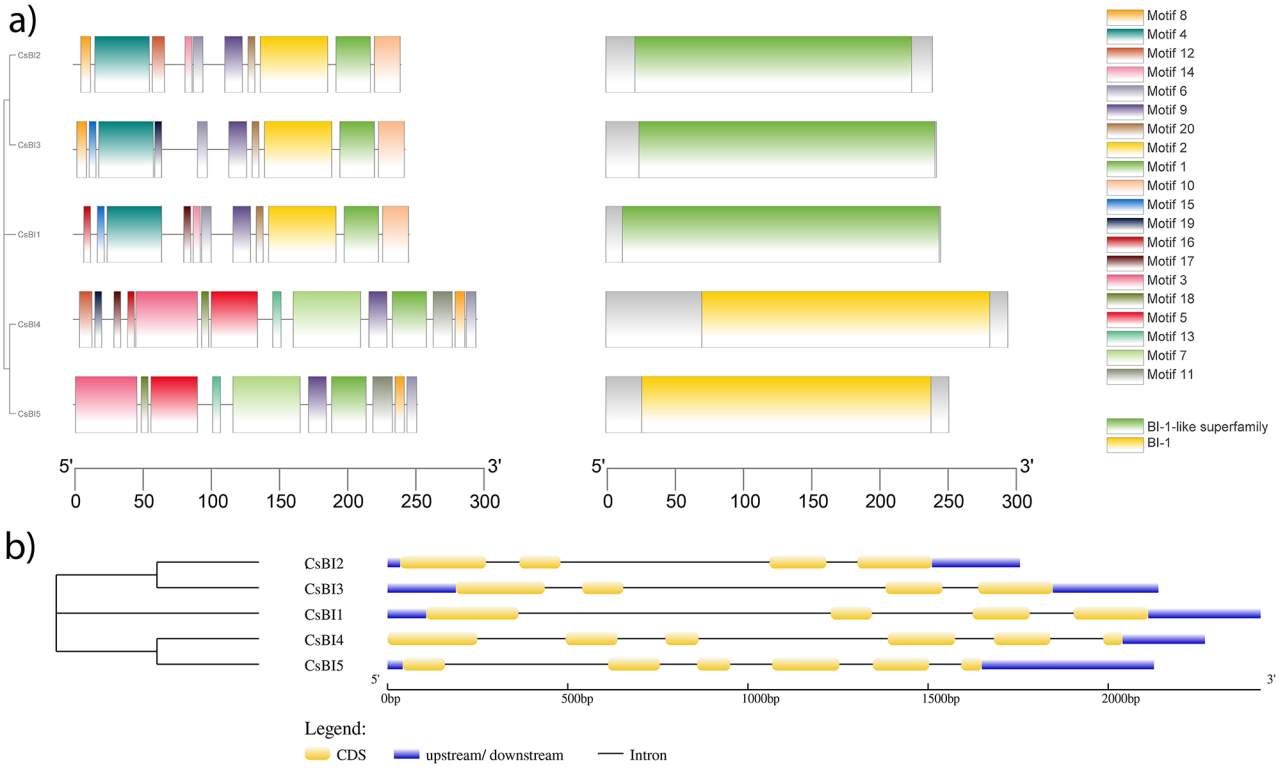

Fig. 3 (**a**) Identification of conserved domains in *C. sativus* through NCBI CDD using the sequence of BAX1-I (**b**) Gene structure and phylogeny of *BI-1* genes from *C. sativus*. Yellow bars represent exons and black lines represent introns in genetic pattern display of the *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4* and *CsBI5* genes

species *C. melo* and *C. moschata*. *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4* and *CsBI5*of *C. sativus* shows syntenic relationships with gene members of their respective groups located on different chromosomes in *C. melo* and *C. moschata*(Fig. 5a, b, c).

Evaluation of *C. Sativus BI-1* genes duplication event

Generally, a Ka/Ks ratio greater than 1 indicates positive selection, a ratio approximately equal to 1 suggests neutral selection, while a ratio less than 1 implies the likelihood of purifying selection, showing minimal functional divergence exhibited by the duplicated genes [74]. The ratio of nonsynonymous mutations (Ka) to synonymous

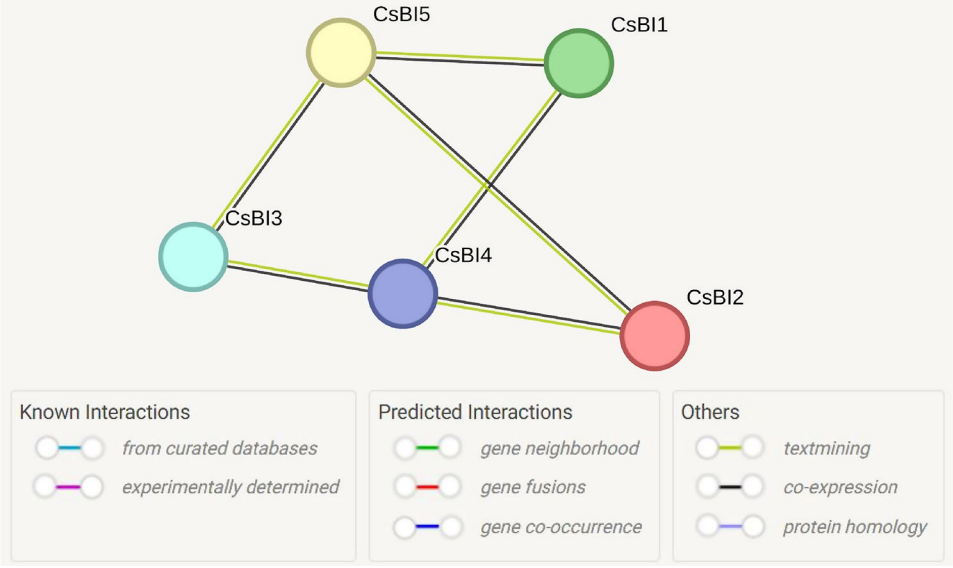

Fig. 4 Protein-protein interactions of *C. sativus* BI-1 proteins, predicted through the STRING database, showed that CsBI1, CsBI2, and CsBI3 proteins are all linked with CsBI4 and CsBI5

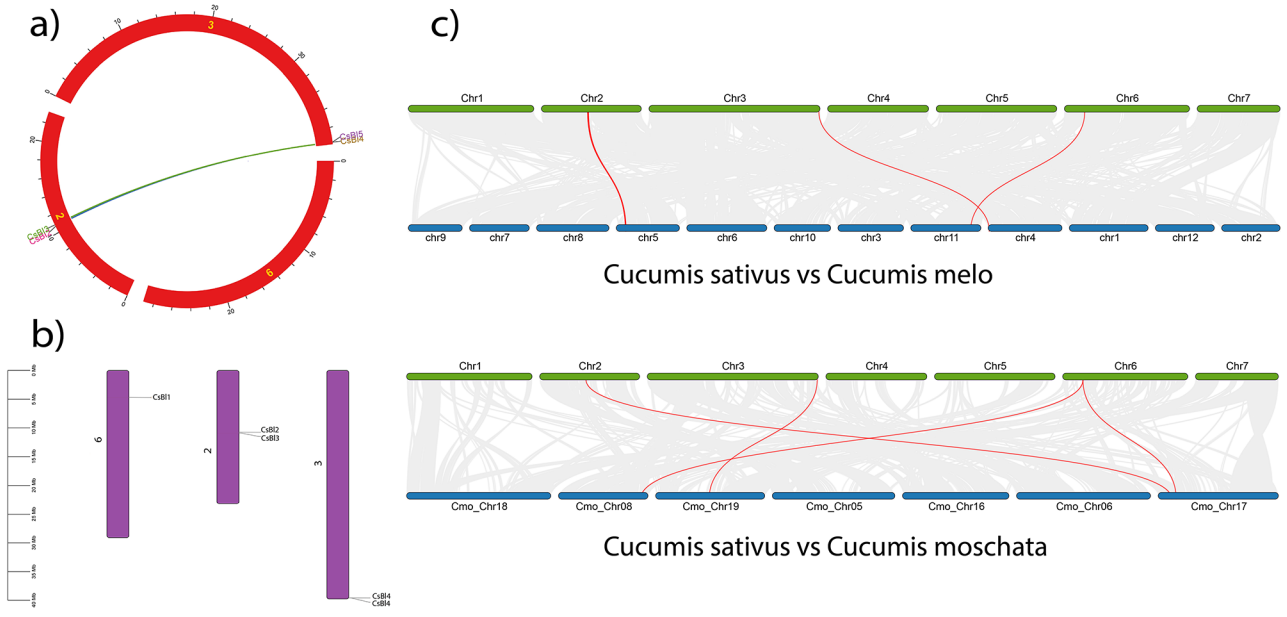

Fig. 5 (**a**) Syntenic relationship of *BI-1* genes within *C. sativus* genome (**b**) Distribution of *BI-1* genes on chromosomes within *C. sativus* genome. Purple bars represented the chromosomes and label on the top of each bar represented the chromosome number. *BI-1* genes were represented in multiple colors on the chromosome of *C. sativus* genome (**c**) Dual synteny of *C. sativus BI-1* genes with *C. melo.* Colored horizontal bars represent chromosomes and label on top *C. sativus* and on bottom *C. melo* and *C. moschata* of these horizontal bars represent the chromosome number of the respective plant species. The orthologue gene pairs are highlighted by red lines and gray lines indicate collinear blocks of *C. sativus* with the genome of *C. melo* and *C. moschata* in the background

mutations (Ks) is denoted by Ka/Ks, varied from 0.092 in the *CsBI2_CsBI3* pair to 0.452 in the *CsBI1_CsBI5* pair. In the paralogous groups of all seven pairs in *C. sativus*, the Ka/Ks ratio was less than 1, recommending a low likelihood of significant functional divergence during the process of duplication, likely by virtue of purifying selection among these paralogous (Fig. 6).

Analysis of *cis*-regulatory elements of *BI-1* genes

Cis-elements are crucial regulatory sequences found in the promoter regions of genes, influencing their expression in response to various environmental and physiological cues. Among the abiotic stress-responsive cis-elements, ABRE (Abscisic Acid-Responsive Element) plays a role in drought and stress responses, while ERE

Seq1_Seq2	Ka	Ks	Ka_Ks	MYA
CsBI1_CsBI5	0.998	2.204	0.453	180.696
CsBI1_CsBI4	0.942	3.328	0.283	272.766
CsBI2_CsBI4	0.944	4.439	0.213	363.866
CsBI2_CsBI3	0.350	3.792	0.092	310.835
CsBI3_CsBI4	0.870	3.129	0.278	256.457
CsBI4_CsBI5	0.080	0.593	0.135	48.639
CsBI4_CsBI3	0.870	3.129	0.278	256.457

Fig. 6 Ks and Ka values for BI-1 gene pairs of *C. sativus* were calculated using the TBtools Simple Ks/Ka Calculator. This evaluation provides insights into the duplication events of *C. sativus* BI-1 genes

(Ethylene-Responsive Element) responds to ethylene signaling. MBS (MYB binding site) is involved in drought-inducibility, and DRE core (Dehydration-Responsive Element) is central to the plant's response to dehydration stress. TCA-element is associated with salicylic acid responsiveness, LTR (Low-Temperature-Responsive Element) is activated under low-temperature stress, and ARE (Anaerobic Responsive Element) is involved in anaerobic conditions. The TCT-motif responds to copper stress, CCGTCC motif is linked to ABA and MeJA responsiveness, STRE (Stress-Responsive Element) is generally associated with various stress responses, and the CAT-box is specific to the catalase gene promoter, involved in oxidative stress responses. On the other hand, among the biotic stress-responsive cis-elements, E2Fb, a cell cycle-regulated element, may be activated during pathogen infection. The Myb-binding site is linked to the response to fungal infections, while AP-1 (Activator protein 1) plays a role in the plant's response to pathogens. TGA-element is associated with salicylic acid signaling and defense against pathogens, while the AE-box is involved in anaerobic induction. The W box is activated in response to physical damage or wounding, and as-1 (Tobacco pathogenesis-related gene 1 element) is related to pathogen defense. The Myb motif is a general Myb binding site that can be involved in defense responses, and motif I has a role in plant defense mechanisms. Lastly, the RY-element serves as a binding site for transcription factors related to pathogen responses. These cis-elements collectively contribute to the intricate regulatory network governing plant responses to both abiotic and biotic stresses. The cis-regulatory elements (CRE) analysis showed that *CsBI1* had CREs such as G-box, ABRE, G-Box, CAT-box, TCA-element, MYB recognition site, O2-site, TGA-element, ABRE3a, ABRE4, ERE, A-box, TGACG-motif and as-1 motif. *CsBI2* had MYC, STRE, MYB, CAT-box, TCT-motif, ACE, I-box, Myb, Myb-binding site and ERE motif. *CsBI3* contained MYC, STRE, MYB, G-box, and ABRE, MYB-like sequence, TCA and WRE3 motif. *CsBI4* possessed MYC, STRE, MYB, G-box, ABRE, G-Box, Myb, Myc, TCCC-motif, and circadian, MYB-like sequence, Wbox, P-box, ERE, A-box, TGACG-motif and as-1 motif. *CsBI5* had MYC, STRE, MYB, ABRE, G-Box, W box, P-box, GARE-motif, Myb-binding site and TCA element motif (Table 3S) (Fig. 7).

Putative miRNA targets in *C. Sativus*

A class of short RNAs (sRNAs) known as microRNAs (miRNAs) attach to target mRNAs (messenger RNAs) at highly complementary locations to suppress the expression of certain genes. miRNAs have played a crucial role in plant life by regulating developmental programmes and carrying out responses to biotic and abiotic stimuli through their complex integration into gene expression programmes [51, 75]. Consequently, a total of 18 miRNAs were discovered, all of which targeted *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4* and *CsBI5* genes. These miRNAs ranged in length from 21 to 23 amino acids. The identified miRNAs were specific and targeted individual genes, although multiple miRNAs targeted a single gene, with the exception of csa-novel-mir77, (the regulation of plant response to salt and drought stresses in an abscisic acid) which targeted two genes [76]. While most of the miRNAs caused inhibition through cleavage, two miRNAs hindered the translation process of their respective targeted genes. 3 miRNAs were targeting *CsBI1*, 3 miRNAs targeted *CsBI2*, 4 miRNAs were targeting *CsBI3*, 6 miRNAs were targeting *CsBI4* and 2 miRNAs were targeting *CsBI5* (Table 4S).

Physiological and bio-chemicals under virus stress

Impact of virus infection on chlorophyll content antioxidant activity

When it comes to chlorophyll concentration between two varieties of cucumber, virus inoculated plants possessed significantly lower concentration of Chl *a*, Chl *b* and total Chl. as compared to control group. However, chlorophyll concentration was found lower in ICS CU-1652 than the other variety. While, the plants with virus inoculum resulted in a substantial increase in the antioxidant activity of both varieties. However; the antioxidant level was higher in the "Oscar" variety of cucumber (Fig. 8).

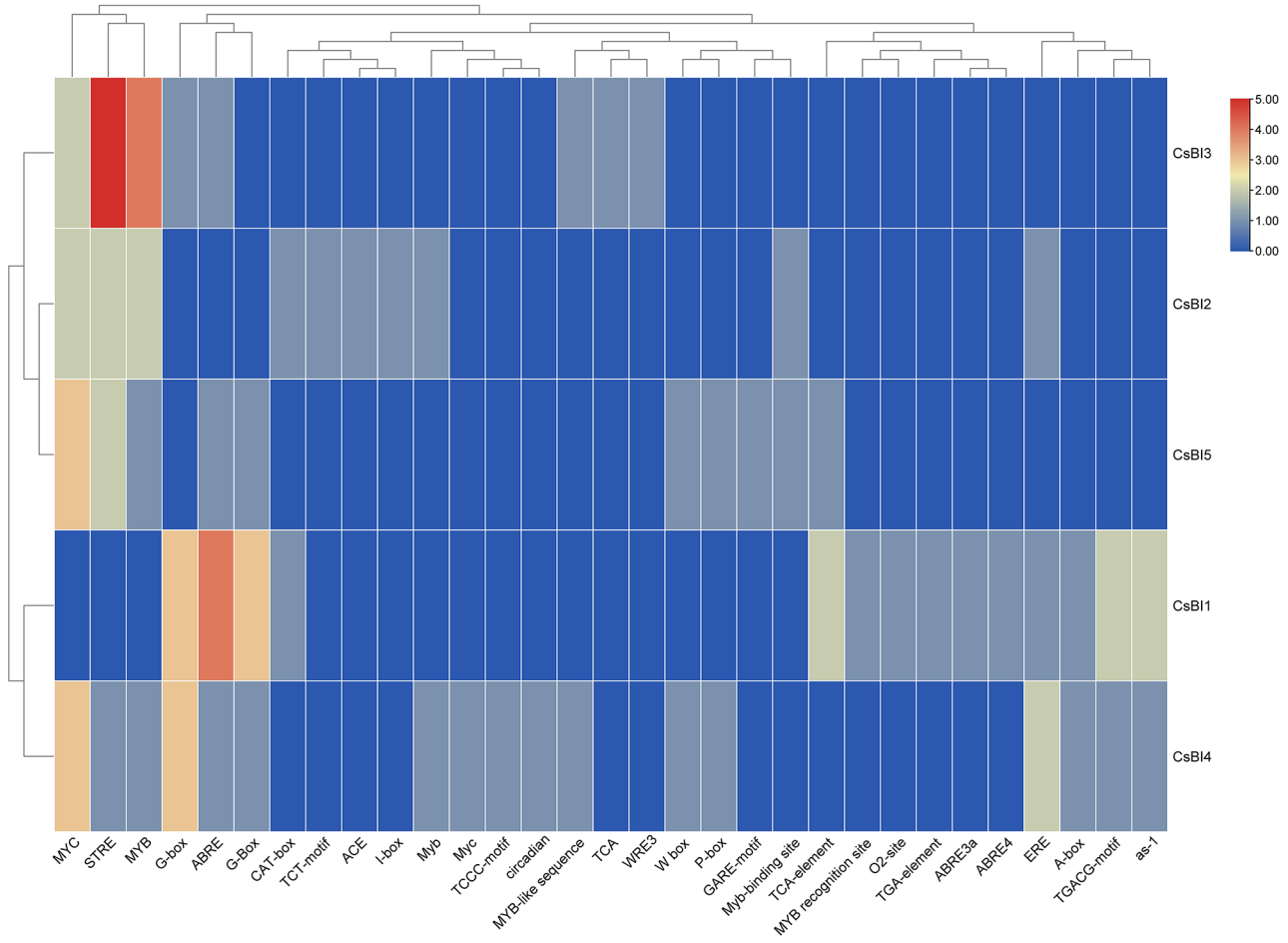

Fig. 7 A heat map representing the cis-elements found in *C. sativus BI-1* genes. The cis-elements are categorized into three groups: phytohormone responses, stress responses, and growth and development

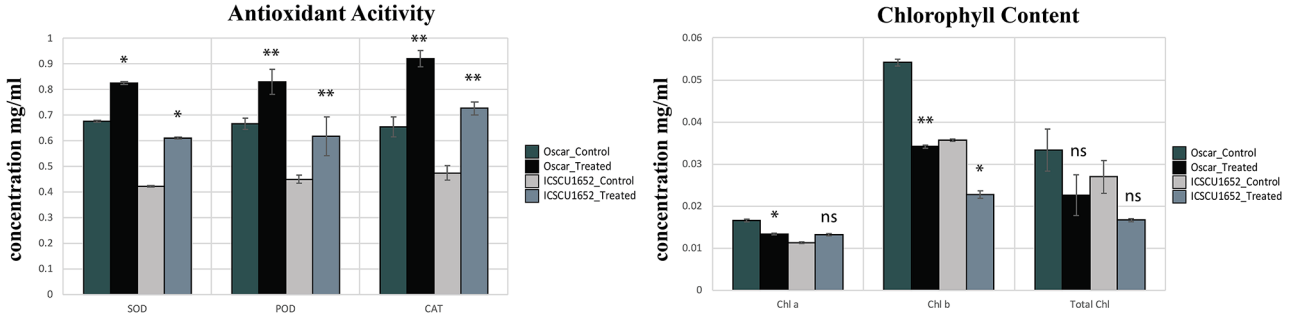

Fig. 8 Graphical representation of the effect of virus infection on chlorophyll content (concentration mg/ml) and antioxidant activity (unit mg/protein) in both plant varieties (Less than 0.05=*, Less than 0.01=**, ns = non significant). Graph was constructed with R programming language. The graphs illustrate how virus infection impacts these physiological parameters, highlighting differences between the two varieties

Validation of gene expression of *BI-1* in response to ToLCNDV by RT-qPCR

The variations in *BI-1* gene expression in two varieties of cucumber were analyzed using real time quantitative PCR after 15 days of infection. After infection with ToLCNDV, expression changes of 5 *BI-1* genes in both varieties of cucumber were observed. The expression patterns of all members of the *BI-1* gene family were notably different. The *CsBI1* gene was found to be up-regulated in response to Begomovirus infection in the "Oscar" variety, as compared to the "ICSCU1652" variety. *CsBI2* showed a slight upregulation compared to the *CsBI1* gene. On the other hand, *CsBI3*, *CsBI4*, and *CsBI5* exhibited a high level of upregulation in the "Oscar" variety under

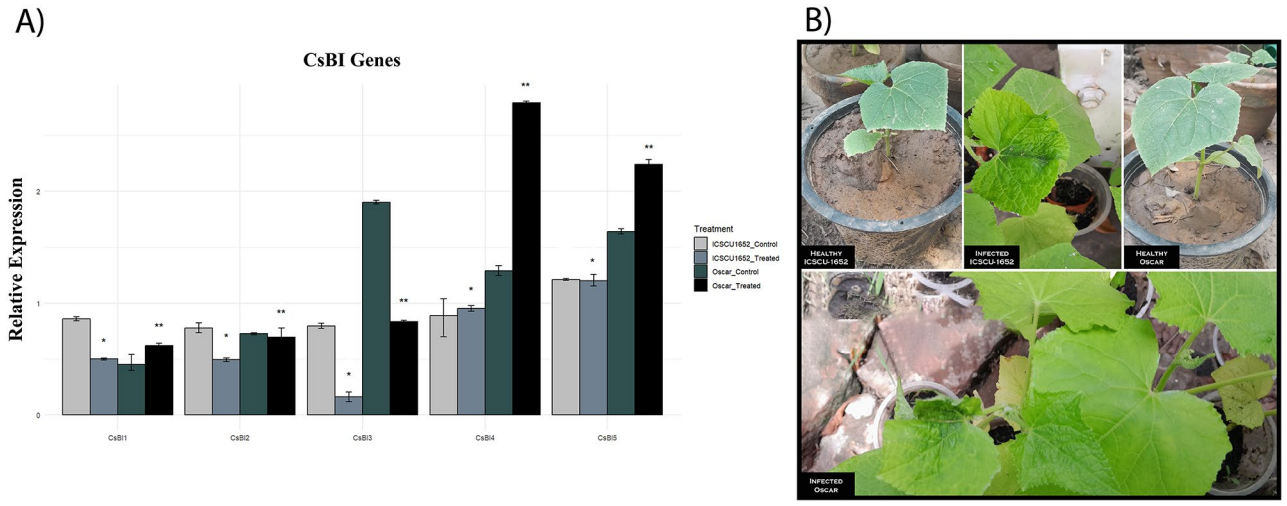

Fig. 9 (**a**) Quantitative real-time PCR analysis of the relative expression pattern of *BI-1* gene family in cucumber leaf after inoculation of *ToLCNDV* (Less than 0.05=*, Less than 0.01=**, ns = non significant). Graph was constructed with R programming language (**b**) Representation of healthy and infected plants of both varieties

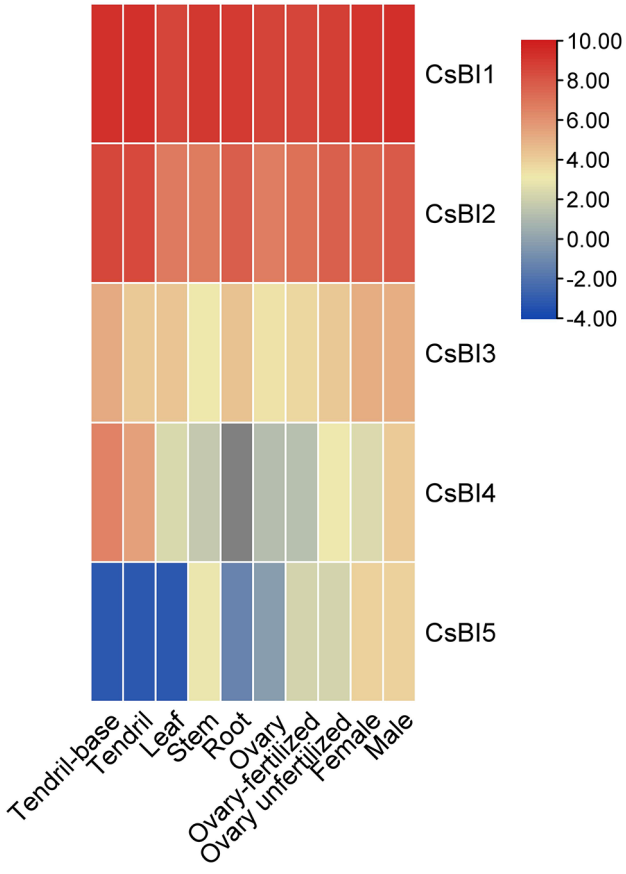

Fig. 10 Expression of the *CsBI* genes in different cucumber tissues. Based on publicly available transcriptome data (PRJNA80169), the transcriptional levels of *CsBI* genes in ten tissues or organs of cucumber Chinese long V2 were examined. Using a heatmap and a log2RPKM value, the expression of *CsBI* genes across the entire genome was displayed. From blue to red, the color scale depicted escalating expression levels

Begomovirus infection. Overall, the results demonstrate that all members of the *CsBI* gene family were significantly upregulated (Fig. 9a, b).

Transcriptomic analysis of *C. Sativus BI-1* genes

Tissue-specific expression analysis

Using the RNA-seq data on the CuGenDB, the tissue-specific expression of the five *CsBI* genes was summarized. Some genes, such as *CsBI1* and *CsBI2*, which were extensively expressed in each tissue, suggested that these *BI-1* genes are crucial for cucumber development. Few tissues had modest levels of *CsBI5* expression. Different tissues had variable levels of *CsBI4* expression; for instance, tendril base, tendril, and male flowers had relatively high levels, whereas other tissues had lower levels. These showed that the *CsBI* genes had various functions during cucumber growth (Fig. 10).

Chitosan oligosaccharide induces cold tolerance

To investigate the beneficial effects of chitosan oligosaccharide against cold stress, cucumber seedlings were exposed to temperatures of 12 °C during the day and 6 °C at night. Seedlings were pretreated with 50 mg L−1 chitosan oligosaccharide, while distilled water served as the control. Samples were collected at 0, 3, 12, and 24 h after cold stress initiation for transcriptome analysis. During the progression of cold stress, the expression levels of CsBI3, CsBI4, and CsBI5 genes were significantly elevated compared to those in the control group (Fig. 11). Conversely, *CsBI1* was significantly downregulated with increasing cold stress, while *CsBI2* was also downregulated but not significantly.

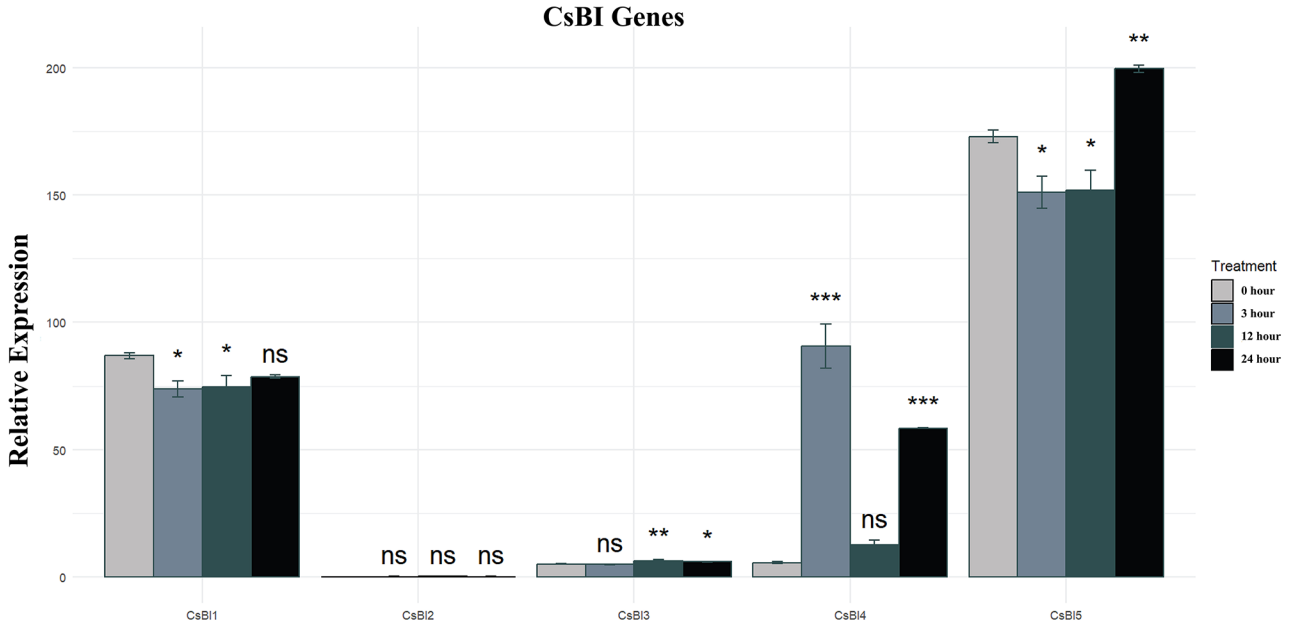

Fig. 11 Graphs representing the expression levels of *C. sativus* BI-1 genes under cold tolerance induced by exogenous chitosan oligosaccharide (Less than 0.05=*, Less than 0.01=**, Less than 0.001=*** ns = non significant). Graph was constructed with R programming language

Discussion

In the post-genomics era, the availability of genomic resources has expanded the possibilities for crop breeding to address the challenges posed by biotic and abiotic stresses, [65, 77] which significantly impact crop growth, development, and the ultimate quality and yield of agricultural products [78, 79]. As Pakistan, is an agrarian nation, it takes advantage of a suitable climate that allows year-round cultivation of vegetables Current study was aimed for a better understanding of genome-wide analysis and involvement of *BI-1* gene for resistance against abiotic and abiotic stress. *BI-1* inhibited cell death and was found in both animals and plants [80]. The mRNA expression of *BI-1* has been studied in different tissues of plants which boost up during aging, biotic and abiotic stresses [81].

In this study, identification and analysis of *BI-1* genes offers crucial insights into their function and evolutionary history [41]. It revealed five distinct genes (*CsBI1*, *CsBI2*, *CsBI3*, *CsBI4*, and *CsBI5*) in cucumber, each encoding proteins of varying lengths and sizes. Comparative phylogenetic analysis, involving *BI-1* genes from cucumber and other species like *A. thaliana*, *C. melo*, *O. sativa*, *T. aestivum*, *B. hispida*, *S. lycopersicum*, and *C. pepo*, highlighted four main clades (1, 2, 3, and 4). These clades underscore different gene lineages and evolutionary divergence among *BI-1* family members: Clades (3and 4) were largest with 23 members of each, clade 1 of 21 members, and clade 2 of 14 members. Each clade member may have similar function with each other. Subcellular localization analysis indicated the localization of these five *BI-1* genes in distinct cellular compartments such as plasmid, Golgi apparatus, vacuole and ER, thus suggesting the potential of BI-1 proteins to play part in various cellular processes [82]. Sub-cellular localization of this gene had previously been studied by various other researchers indicating its presence in distinct cellular compartments particularly in ER and *BI-1* was found conserved across eukaryotes [83]. The maximum localization of cucumber and tobacco BI-1 proteins in the plasma membrane and vacuoles suggests that the BI-1 gene plays a role in regulating processes within these cellular structures. Specifically, this means that the BI-1 gene may be involved in functions related to the plasma membrane, such as signal transduction, ion transport, and interaction with the external environment, as well as in vacuoles, which are important for storage, waste disposal, and maintaining cellular homeostasis. The high localization of BI-1 proteins in these areas indicates their potential importance in maintaining cellular stability and responding to stress conditions in cucumber and tobacco [42].

The non-redundant BI-1 protein sequences from *C. sativus* reveal highly conserved domains. Each gene in *C. sativus* maintains its conserved domain. Notably, Motif 1 is present in all genes, suggesting its potential functional role in encoding the BI-1 protein. While *CsBI1*, *CsBI2*, and *CsBI3* genes share certain conserved motifs, *CsBI4* and *CsBI5* possess their unique set of motifs. However, despite these variations, all five BI-1 genes maintain the BI-1 domain, underscoring its conservation across them [82]. Gene structure analysis of cucumber's *BI-1* genes

indicates variability in the number of introns, ranging from 3 to 5. By aligning exons and introns, researchers gain insights into the evolutionary history and interrelationships among these genes in *C. sativus* [84]. This alignment not only reveals the fundamental gene structures but also provides valuable information on the evolutionary associations between genes in different organisms [85].

Protein-protein interaction network analysis of cucumber BI-1 proteins revealed that CsBI1, CsBI2, CsBI3, CsBI4, and CsBI5 demonstrated interactions with themselves within the *C. sativus* genome (Fig. 4). This indicated that CsBI1 proteins were linked with CsBI4 and CsBI5, while CsBI2 proteins also exhibited linkages with CsBI4 and CsBI5. Similarly, CsBI3 proteins were connected with CsBI4 and CsBI5. These interactions suggested that these proteins might have had similar functions in cucumber [84]. These interactions reflect crucial functions and processes in plants, including signal transduction pathways, plant development, physiology, and responses to pathogens [86]. Analysis of chromosomal location indicates that CsBI1 is situated on chromosome 6, while CsBI2 and CsBI3 are both located on chromosome 2. Similarly, CsBI4 and CsBI5 are positioned on chromosome 3. This data offers insights into the genomic organization and distribution of BI-1 genes within the cucumber genome. Furthermore, studying the chromosomal location of genes facilitates the investigation of gene duplications and their evolutionary implications [87].

Genes located on the same chromosome are likely the result of tandem duplication, indicating the presence of two or more copies of the gene [88]. Conversely, when genes are found on different chromosomes, they may have arisen from segmental duplication, which involves the duplication of genetic segments across chromosomes [89].

Synteny analysis revealed that *BI-1* genes in cucumber have undergone tandem duplications. Specifically, *CsBI2* is genetically linked with *CsBI3*, and *CsBI4* is linked with *CsBI5*. The *CsBI2* and *CsBI4* genes are located on chromosome 2, whereas *CsBI3* and *CsBI5* are located on chromosome 3. Synteny acts as a structural framework that helps for the determination of preserved homologous genes and their particular order across genomes of various species [90]. The analysis of dual synteny indicates that segmented duplication is responsible for the presence of syntenic relationships among all the BI-1 genes in *Cucumis sativus*, *Cucumis melo*, *and Cucumis moschata*. Specifically, *CsBI1*, *CsBI2*, *CsBI3*, *CsBI4*, and *CsBI5 in C. sativus* exhibit syntenic connections with genes belonging to their respective groups located on different chromosomes in C. *melo and C. moschata*.

All 7 paralogous pairs of *BI-1* had Ka/Ks ratio less than 1 indicating that these genes had passed through strong purifying selection pressure and positive selection might have acted on only few sites during the process of evolution among these paralogs [91]. Gene duplication plays a pivotal role in the emergence of new gene subfamilies within genomes and genetic systems during evolutionary events. The emergence of novel gene families primarily occurs through mechanisms such as tandem duplication, polyploidy, and segmental duplications [92]. These duplication events lead to the creation of additional copies of genes, providing raw material for evolutionary innovation and adaptation [93]. Tandem duplication involves the consecutive duplication of genes on the same chromosome, resulting in gene clusters. Polyploidy involves whole-genome duplication events, leading to an increase in the number of copies of all genes within an organism [94]. Segmental duplications involve the duplication of chromosomal segments, contributing to the expansion of specific gene families [95]. Together, these mechanisms drive the diversification and evolution of gene families, enabling organisms to adapt to changing environments and ecological niches over time [96].

The cis-regulatory elements analysis of *BI-1* genes unveils insights into their spatial and temporal expression patterns [97]. Various cis-regulatory elements (CREs) present within the promoter regions of *CsBI* genes are associated with specific responses and signaling pathways. Cis-elements were grouped into phytohormone responses, stress responses, and growth and development. Among the elements associated with growth and development, numerous motifs are prevalent in promoter regions, including the Skn-1_motif, GCN4_motif, MRE, Box-4, CAT-box, O2-site, and circadian elements. Regarding phytohormone responses, ABRE, P-box, TGACG motif, TCA-element, and CGTCA motif are identified, associated respectively with salicylic acid (SA), abscisic acid (ABA), ethylene, and MeJA responses. Furthermore, various stress-responsive elements including ARE, LTR, MBS, and W box correlate with light stress, cold stress, heavy metal, drought and biotic stress responses, providing insights into the regulatory mechanisms enhancing the *CsBI* gene family's responses to abiotic and biotic stress. For instance, the presence of Myb-binding sites suggests involvement in fungal infection responses, while AP-1 sites indicate participation in pathogen response mechanisms. TGA elements are linked to salicylic acid signaling and pathogen defense, whereas AE-boxes are associated with anaerobic induction [92, 93]. Overall, the arrangement and presence of these CREs within the promoter region influence gene expression patterns. In silico analysis of CREs aids in estimating the potential functions of genes based on their regulatory elements [98]. These cis-elements collectively

contribute to the intricate regulatory network governing plant responses to both abiotic and biotic stresses.

Analysis of putative miRNA targets of *BI-1* genes in cucumber suggests that these genes may undergo post-transcriptional regulation by miRNAs. This implies that miRNAs may play a role in the expression levels of *BI-1* genes by targeting their mRNA transcripts for degradation or translational repression. Such post-transcriptional control mechanisms mediated by miRNAs are important for regulating gene expression and maintaining cellular homeostasis in response to various developmental and environmental cues [99]. The interaction between miRNAs and BI-1 can restrict expression levels and potentially alter the function of BI-1 proteins in cucumber. miRNA, the fundamental regulators in plants, play a key part in different biological processes, growth, development, defense and regulation of internal equilibrium [100]. These small RNA molecules display an exceptional conservation across distinct species showing that each microRNA executes its specified task disregarding of the organism it belongs to [100]. Both types of miRNAs have been identified, such as csa-novel-mir97 and csa-novel-mir109, which play roles in regulating plant responses to salt and drought stresses through the abscisic acid pathway [101]. In addition, csa-miR2950 is involved in floral development in the context of biotic stress [102], csa-miR58 plays a major role in enhancing the production of antifungal compounds to bolster the plant's resistance to pathogen infections [103], and csa-miR68 is crucial for the development of the plant's immune system [104].

Virus-stressed plants exhibited high levels of antioxidant activity. Our findings align with previous data, showing increased levels of antioxidants such as superoxide dismutase, peroxidase, and catalase [105]. Catalase reduces the harmful effects of toxic peroxides, while superoxide dismutase decomposes superoxide radicals [60]. The increase in antioxidant enzymes likely results from the activation of plant defense mechanisms under stress [106].

Begomovirus-infected cucumber plants were found to have lower concentrations of chlorophyll a, chlorophyll b, and total chlorophyll compared to the control group. Virus-infected plants showed a significant decrease in photosynthetic pigment concentration [107], which corresponds to decreased photosynthetic activity due to the down-regulation of specific genes during the viral attack [108, 109]. The RT-qPCR study revealed that the expression pattern of *BI-1* genes was up regulated in ToLCNDV-infected cucumber, indicating their involvement in defense responses against both biotic and abiotic stresses. This finding underscores the potential role of *BI-1* genes in mediating plant defense mechanisms in response to pathogen invasion. Furthermore, the role of *BI-1* gene expression in defense responses has been extensively studied in other plant species such as *A. thaliana*, barley, and rice, suggesting its evolutionary conservation across different plant taxa. This suggests that *BI-1* genes may play a crucial role in modulating plant immunity and stress tolerance across a wide range of plant species [80]. In *A. thaliana*, the *AtBI-1* expression is quickly boosted up when subjected to injuries or pathogens [110]. Additionally, the expression study revealed that the *BI-1* genes control the growth of cucumber stem, leaf, and fruit, demonstrating a direct connection between the *BI-1* gene family and the development of cucumber.

During our transcriptomic analysis, comparing the control group with the treatment group revealed distinct trends in gene expression levels under cold stress and chitosan oligosaccharide (CsBI) treatment in cucumber seedlings. Notably, *CsBI3*, *CsBI4*, *and CsBI5* consistently showed significant increases in expression levels in the treatment group, suggesting CsBI's potential to potentiate gene upregulation under cold stress. Conversely, *CsBI1* and *CsBI2* displayed less pronounced downregulation in the treatment group. The gene expression analysis of *BI-1* genes under cold tolerance caused by exogenous chitosan oligosaccharide gives understanding about how these genes respond to abiotic stress thus suggesting that *BI-1* genes may have a functional role against cold stress in cucumber. Additional studies are required to reveal the function of *BI-1* genes in mechanisms that are related to cold stress. The collective findings of the whole study indicate that the *CsBI* gene family plays a highly significant role in both biotic and abiotic stress responses. This study paves the way for enhancing cucumber crop performance under stressful conditions.

Conclusion

The study provides a comprehensive understanding of the *BI-1* gene family in *C. sativus*, including gene structures, phylogenetic relationships, chromosomal locations, and expression patterns. Five *BI-1 genes* were identified and categorized, with conserved gene structures observed across evolution. *CsBI3*, *CsBI4* and *CsBI5* showed high expression levels during cold stress, indicating their importance in abiotic stress defense. Up-regulation of *BI-1* genes in ToLCNDV-infected cucumber suggests their involvement in defense against both biotic and abiotic stresses. Overall, this research enhances our understanding of the molecular mechanisms regulating stress responses in *C. sativus*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10704-5.

Supplementary Material 1

Acknowledgements
The authors extend their appreciation to the Deanship of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom and Department of Plant Breeding and Genetics, Faculty of Agricultural Sciences, University of the Punjab, P.O BOX. 54590, Lahore, Pakistan for funding this work.

Author contributions
The contributions of the authors to this article are as follows: SA played a significant role in data curation, formal analysis, and drafting the original manuscript. MZH and AS were responsible for conceptualization, data curation, methodology development, and co-authored the original draft. MS and RS were actively involved in data curation, formal analysis, software development, supervision, and contributing to the original draft. SA, MAJ, and JT participated in data curation, investigation, methodology development, and played crucial roles in reviewing and editing the manuscript. HA led the efforts in securing funding, contributed to methodology development, provided essential resources for the study, and co-authored the original draft. Finally, JA, BJ, RSL and BUN focused on reviewing and editing the manuscript, enhancing the methodology, and providing additional resources to support the research.

Funding
Not applicable.

Data availability
I affirm that all necessary data and permissions have been provided for this study. Any interested researchers can access the required data to support the findings and conclusions of this article. For publicly archived datasets, hyperlinks are provided in this manuscript in appropriate place for convenience. Rest assured, the plants were collected from Departments of Horticulture Punjab University Lahore with voucher HDpu7598 and submitted to Horticultural bank Punjab University Lahore. I have ensured that all data, materials, software applications, and custom code supporting the claims made in this article are in full compliance with field standards. It's important to note that I have taken into account the possibility of individual journal policies regarding research data sharing, considering the norms and expectations of our discipline.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Data transparency
Rest assured, I have ensured that all data, materials, software applications, and custom code supporting the claims made in this article are in full compliance with field standards. It's important to note that I have taken into account the possibility of individual journal policies regarding research data sharing, considering the norms and expectations of our discipline.

Competing interests
The authors declare no competing interests.

Received: 24 January 2024 / Accepted: 12 August 2024
Published online: 04 November 2024

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