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RESEARCH ARTICLE

Molecular cloning and *in-silico* characterization of gene encoding eukaryotic translation initiation factor (*eIF4E*) from *Capsicum chinense* Jacq.

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Abstract

Capsicum chinense Jaqc., is well known for its high capsaicin content and medicinal properties. The productivity of this crop is extensively hindered by different viruses, among which potato virus Y (PVY) is one of the most devastating viruses worldwide. The interaction between eukaryotic translation initiation factor 4E (*eIF4E*) and potyvirus VPg (viral protein genome-linked) correlates with potyvirus infectivity. Cloning and characterization of the *eIF4E* gene related to Capsicum viral disease resistance is critical for understanding plant-pathogen interactions, especially with potato virus Y infection. The present study aims to isolate, clone, and characterize the full-length gene encoding *eIF4E* from *C. chinense* Jaqc. The full-length *eIF4E* gene sequence obtained was 692 bp (GenBank Accession No.: MN661348) with an ORF of 687 nucleotides and 228 amino acid residues and blast homology analysis of the Cc*eIF4E* sequence showed 99% sequence similarity with *C. annum* and *C. baccatum*. A conserved domain was identified from a CDD search. Secondary structure prediction showed 11 alpha helices and seven beta strands. Phylogenetic analysis showed a higher identity to *C. annum,* indicating the molecular evolution of the Cc*eIF4E* gene. The presented work will pave the way for reverse engineering for the development of viral resistance in Bhut Jolokia.

Keywords: *Capsicum chinense* Jacq, eukaryotic translation initiation factors (*eIF4E*), cDNA, cloning, PVY, structure-function.

Introduction

Bhut jolokia (*Capsicum chinense* jacq),of the *Solanaceae* family, one of the hottest chili peppers in the world, is an indigenous cultivar of North-East India and is widely consumed throughout the world. This crop has a huge commercial potential in both the domestic and foreign markets because of its strong pungency and aroma. The government of Assam and other Indian north-eastern states are pursuing a number of initiatives to increase the production of Bhut jolokia. Production of Bhut jolokia has been increasing every year in north-eastern India (Meetei et al. 2016). Over a 1,050-ha area, 3,420 MT of Bhut jolokia were produced in 2017 (Malakar et al. 2019). There are no latest data available for Bhut jolokia production after 2017. This important cash crop is vulnerable to several devastating biotic stresses caused by viruses, fungi and bacteria. Among these, viral diseases caused by potato virus Y (PVY), groundnut bud necrosis virus (GBNV), chilli leaf curl virus (ChLCV) and cucumber mosaic virus (CMV) have been a serious constraint to *C. chinense* production in India, leading to extensive economic harm to the region and the nation. PVY is a type species of the genus potyvirus and is the most devastating virus of Bhut jolokia. The highest incidence has been recorded up to 87.5% (Talukdar et al. 2017). The presence of five PVY strains (PVYN, PVYO, PVYC, PVYN-Wilga, and PVYNTN) was confirmed by screening Bhut jolokia germplasms using the DAS-ELISA method. The family Potyviridae contains a monopartite single-stranded

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positive-sense RNA genome with a covalently bound viral-encoded protein (VPg), which is attached at the 5′ terminus and a 3′poly-A tract. The genome is about 10 kb in length and is translated into polyprotein and then subsequently cleaved by viral encoded proteases into small polypeptides. In plants such as *Arabidopsis thaliana*, peas, pepper, tomato and lettuce, the eukaryotic translation initiation factors eIF(iso)4E and *eIF4E* play crucial role in potyviral infection (Lellis et al. 2002; Kang et al. 2005; Duprat et al. 2002; Nicaise et al. 2003; Ruffel et al. 2005; Yoon et al. 2020; Zlobin et al. 2023 and Carrington et al. 1992). The core of the eIF(iso)4F complex is formed by the interaction between mRNA cap-binding protein eIF(iso)4E and scaffolding protein eIF(iso)4G, and initiates the translation from mRNAs with unstructured 5′ leaders (Gallie et al. 2001). The *eIF4F* complex is composed of *eIF4E* and *eIF4G* and regulates the translation initiation of mRNAs with complex 5′ structures (Gallieet al. 2001b). Since the potyviral RNA translation is mainly dependent on host translation factors, including *eIF4E,* which interacts with the central domain of the VPg protein, thus the abolishment of either *eIF4E* gene or its isoform in Bhut jolokia helps develop PVY virus-resistant plants without causing changes to the plant genome, this can be considered to be in safer side for human consumption and for release into the surrounding environment. However, *eIF4E,* which is a vital gene responsible for viral infection, has never been cloned and characterized from Bhut jolokia till date. The present study documents the molecular cloning and *in-silico* characterization of *eIF4E* as a first step in understanding the structure and the role of *eIF4E* in the viral translation process in the important cash crop *C. chinense* Jacq.

Materials and methods

RNA preparation

Healthy seedlings of local Bhut jolokia (*Capsicum chinense* Jacq.) that produce red colored pods were collected from the Horticultural orchard of Assam Agricultural University, Jorhat-785013, Assam and grown in pots. The potted plants were maintained in a net house. Tender leaves (0.5 g) were collected for RNA isolation at 4/5 leaf stage of the seedlings, crushed in liquid nitrogen, and total RNA was isolated following the standard protocol of TRI Reagent®. The RNA was electrophoresed in 0.8% agarose gel with tris-borate EDTA (TBE) buffer at 5v/cm for 3 hours. RNA yield was checked in a spectrophotometer (NanoDrop1000, Thermo-scientific) with absorbance at 260 nm.

Cloning of CceIF4E

Four pairs of degenerated primers have been designed considering the most conserved region of known *eIF4E* (Supplementary Table 1). The conserved region of *eIF4E* gene from Bhut jolokia was amplified using Takara's PrimeScript™cDNA (complementary DNA) Synthesis Kit as described in the manufacturer's guidelines. The amplified product was successfully cloned into pGEM®-T Vector (Promega) and then introduced into *E. coli* strain JM109 followed by colony PCR and sequencing (ABI 3500 Sequencer). BLAST analysis confirmed that the amplified sequence is the conserved region of*eIF4E*. Based on the generated sequence, gene-specific primers have been designed (Supplementary Table 2) and 5′ Rapid Amplification of cDNA Ends (5′-RACE) and 3′-RACE PCR were performed. The full-length cDNA synthesis was performed using Invitrogen's GeneRacer Kit with SuperScript III RT, following the protocol as described in Kalita et al. (2015). The RACE PCR products (5′ and 3′) were then cloned into a pCRTM4-TOPO vector (Invitrogen, Life Technologies) and followed by transformation into *E. coli* strain JM109. The ligation of the insert into the vector was confirmed by colony PCR. The amplified full-length cDNA sequence has been submitted to the GenBank database.

ORF prediction

The DNA sequence was converted to primary protein sequence using ORF (Open Reading Frame) Finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) and the converted sequence has been used for protein analysis. The minimum ORF length has been set to 75 and ATG codon was considered as the start codon for the ORF translation. For domain analyses, the CD-Search tool within NCBI (Wang et al. 2022) has been used for domain prediction in the cds sequence. The search database CDD V3.15-48963 PSSMs was used with an expected value of 0.010000.

Phylogenetic analysis

Phylogenetic analysis of the Cc*eIF4E* sequence was carried out considering the blast result of NCBI. A total number of hit sequences were used for the multiple sequence alignment and then phylogenetic analysis was performed using neighbor-joining method in MEGA11 tool. The bootstrap value for the analysis was set to 100. For the identification of a set of homologous sequences and to download the identified sequences, MEGA's own browser built on top of the Google Chrome toolkit, was implemented. For alignment of the downloaded homologous sequences, ClustalW (Thompson et al. 1994) and MUSCLE algorithms of MEGA version 6.0 (Tamura et al. 2021) were used. A phylogenetic tree using the aligned sequences has been constructed in MEGA.

Structure analysis and validation

The protein sequence of Cc*eIF4E*(*C. chinenseeIF4E*) was used for the prediction of tertiary structure in RaptorX structure prediction server. C*eIF4E* protein model was visualized in PyMOL (Janson et al. 2021). The validation of the model structure of *eIF4E* TF was performed in RAMPAGE (Zhang et al. 2019) and ProSA server (Wiederstein et al. 2007).

Molecular dynamics simulation

The molecular dynamics study presented in the current work was conducted in the AMBER18 software package (Case et al. 2005) and ff14SB force field with TIP3P water model. The modeled structure was neutralized by adding counter-ions closer to the solute surface. Particle Mesh Ewald (PME) was exploited for considering the long-range interactions. The modeled structure was minimized with 1000 steps of steepest descent and then 2000 steps of the conjugate gradient procedure at 50 ps of heating and 50ps of density equilibration. The modeled structure was equilibrated over 50 ns. Shake algorithm was used for constraining all the bonds and the time step of the simulation was set to 2fs. The Berenson thermostat was used for controlling the temperature. The temperature of the simulation was set to 300 K and pressure was set to 1 atm.

Results

Cloning of full-length CceIF4E cDNA

Total RNA from younger leaves of Bhut jolokia yielded two intact bands of RNA without any DNA contamination (Fig. 1A) and the RNA yield was 2.8 µg/µL. The core region of Cc*eIF4E* was amplified by using four pairs of degenerate primers, which were designed based on conserved sequences of *eIF4E* from the public database. The amplified fragments were sequenced and the sequence amplified by primer pair four was considered (Fig. 1B) for designing the gene-specific primers to further amplify the 5′ and 3′ end of DNA fragments. The 5′ RACE product was 400 bp and the 3′ RACE product was of 500 bp (Fig. 1C). The sequences obtained from RACE primers and degenerate primer were assembled and the obtained full-length cDNA of Cc*eIF4E*was cloned. Confirmations of successful recombinant clones were assured by colony PCR. Results of colony PCR showed that seven randomly selected colonies gave a band size of expected length (~700 bp) (Fig. 1D) and the purified PCR product was sequenced. The sequence obtained was 692 bp (GenBank Accession No.: MN661348) with ORF of 687 nucleotides and 228 amino acid residues. BLASTP and Multiple sequence alignment revealed the protein homology among the *Capsicum* and *Solanum* species and identified the conserved motif of 20 amino acids long with a consensus sequence of MAxAEMERTxSFDxAxKLKA (marked within a blue box) in the N-terminal end (Fig. 2A). The figure also revealed that the amino acids are highly conserved among C. *chinensis,* C. *annum* and C. *baccatum*. Trypsin contains a nucleophilic residue Ser in the enzyme active site which attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate.

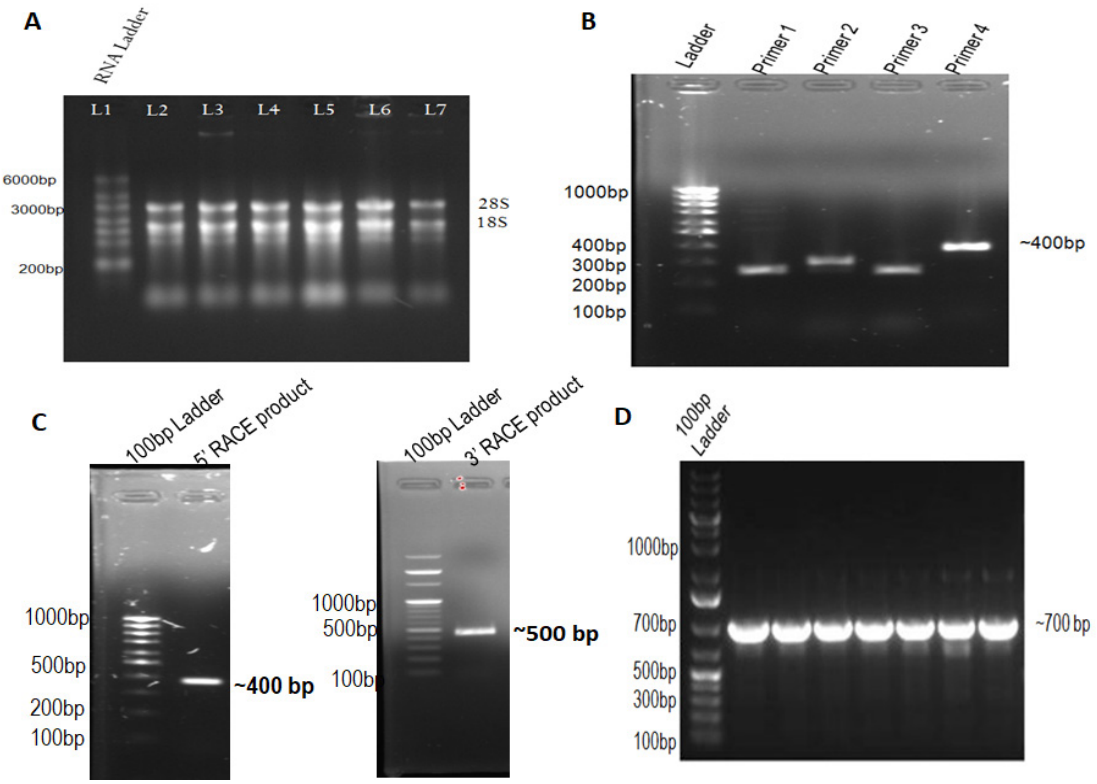

Fig. 1. (A) Agarose gel electrophoresis showing total RNA from tender leaves of Bhut jolokia, (B) The core region of Cc*eIF4E* was amplified using four pairs of degenerate primers, (C) Amplification of C*eIF4E* gene with 5′RACE and 3′RACE primers, (D) Colony PCR products showing 7 randomly selected colonies with a band size of expected length (~700 bp)

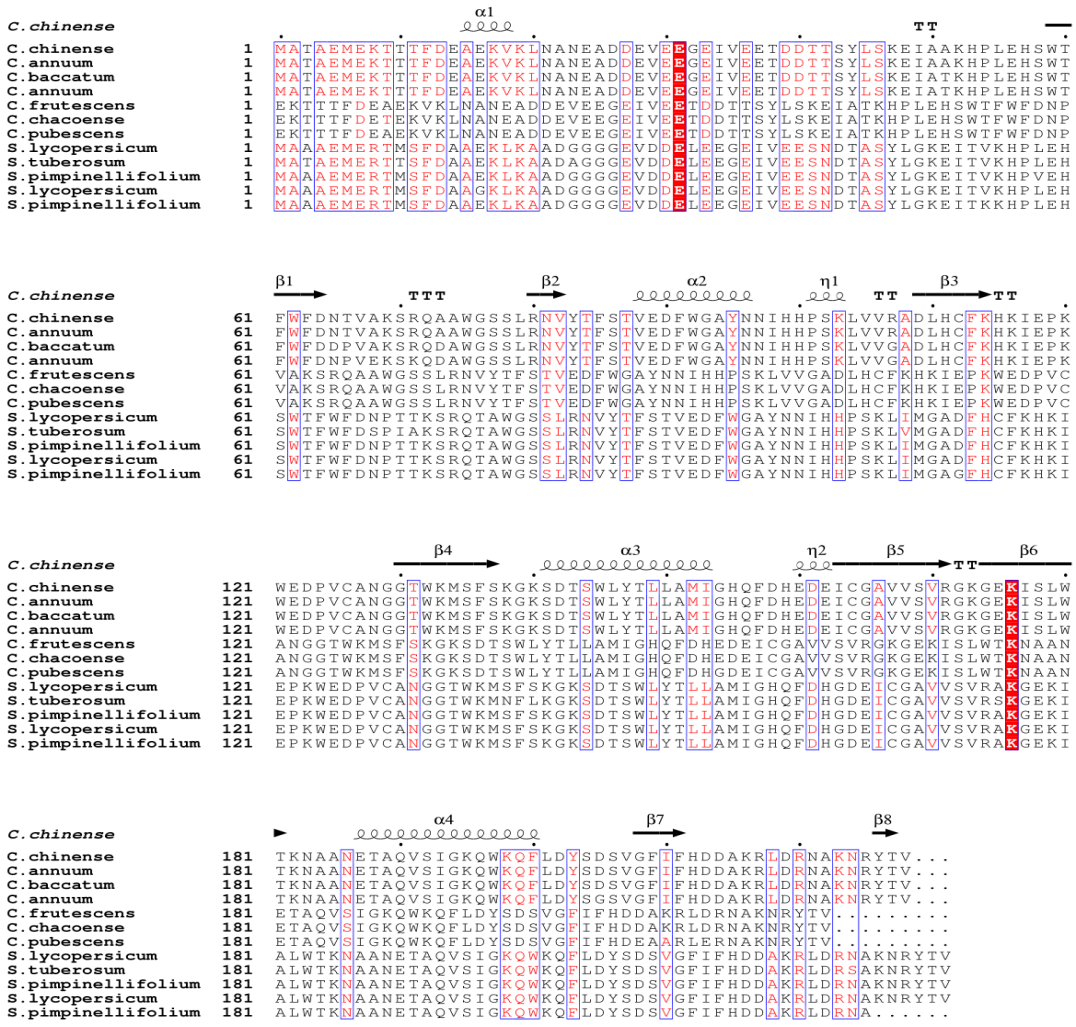

Fig. 2. A. Based on the results of BLASTP, the best templates, along with the EIF4E sequence, were multi-aligned with the ESPRIPT tool to identify the presence of conserved motifs

Domain analysis

Only one domain was found in the coding sequence, i.e., eukaryotic initiation factor 4E from 162-635 with E-value 6.49e-70. The *eIF4E* has a crucial role in the initiation of protein synthesis on capped mRNAs in the cytoplasm. The *eIF4E* has been found to have additional functions, namely translation of some uncapped viral RNAs, roles in aging and the nuclear export of specific mRNAs.

Phylogenetic analysis

A homology blast showed that Cc*eIF4E* is substantially homologous to other eukaryotic translation initiation factors 4E from *C. annum* and *C. chinense*, with a sequence identity of 95%. Neighbour-Joining method was used for inferring the evolutionary history. The phylogenetic tree has been drawn to scale with the same units of branch lengths as the evolutionary distances which were computed using the Maximum Composite Likelihood method and were presented with the units of the number of base substitutions per phylogenetic analysis. The *eIF4E*has clustered with the *C. annum* and *C. baccatum* subgroups (Fig. 3A).

Protein structure prediction and validation

The input has been predicted as two domain(s) and the best template was found to be2wmcA with *p-value* of 2.38e-09. Overall uGDT (GDT) was 164 (72%), 177 (77%) residues were modeled, 67 (29%) positions were predicted as disordered. A total of 22% H, 20% E and 57% C has been found for the secondary structure of the enzyme. The solvent access in the enzyme sequence was found to be 49% E, 23% M and 26%B. Structural validation for the 3D structured model was constructed using the Ramachandran plot in the RAMPAGE server and PyMOL. It has been observed that the modeled structure of *eIF4E* sequence has 98% of residues in the most favored region and 2% in the allowed region (Fig.3C) and the structure prediction showed the presence of 11 alpha helices and 7 beta strands and also the presence of Trp and Ser residues (Fig. 3B). Protein folding energy and quality of

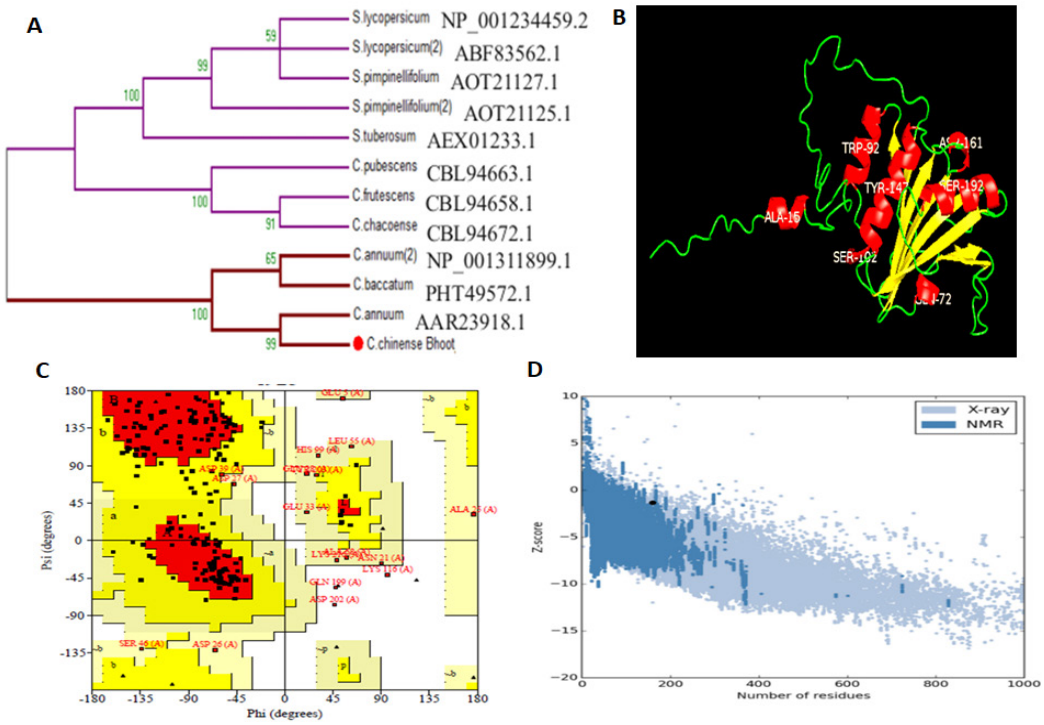

Fig. 3. (A) Phylogenetic tree constructed in MEGA showing *eIF4E* clustered with the C. *annum* and C. *baccatum*, (B) 3D protein structure model of *eIF4E* sequence (PyMOL) was predicted by RaptorX structure prediction server and validated by constructing Ramachandran plot using RAMPAGE server, (C)The modeled structure of *eIF4E* sequence has 98% residues in the most favored region and 2% in allowed region, and (D) protein folding energy of the model structure was validated using ProSA server with the quality index represented by Z-score (-2.7)

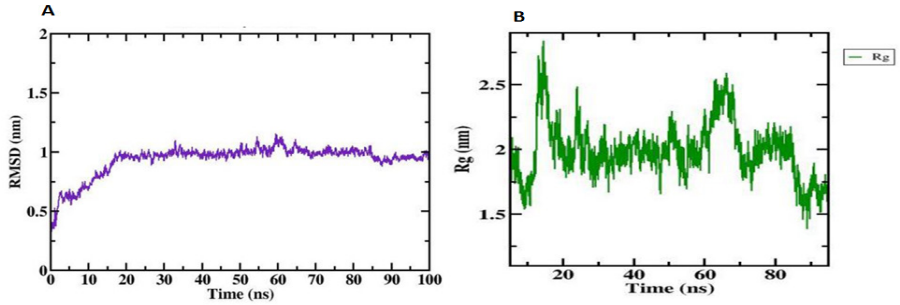

Fig. 4. (A)The RMSD plot confirmed that the modeled structure of *eIF4E* underwent rapid structural changes and reached equilibration after 25 ns of the simulation period; conformational dynamics of the modeled structure of Cc*eIF4E* sequence were analyzed by MD simulation up to 100 ns, (B) Radius of gyration of peptide backbone was a function of time, and has oscillated to a greater degree before 20 ns, confirming that the structural convergence and folding occurred after 20 ns from the simulation being initiated

the model structures has been validated in ProSA server and the quality index was represented by Z-score (-2.7) (Fig. 3D).

The conformational dynamics of the modeled structure of Cc*eIF4E* sequence were analyzed by MD simulation up to 100 ns. The conformational stabilities of the modeled structure were investigated by calculating the root-mean-squared deviation (RMSD) with reference to the initial energy minimized and equilibrated structure. The backbone RMSDs of the peptide have been observed to deviate up to 25 ns and then the structure is converged and stable conformation is attained (Fig. 4A). Rapid structural changes of the modeled structure, confirmed by the RMSD plot, finally reached equilibration after 25 ns of the simulation period. The radius of gyration was measured to quantify its compactness and the mass-weighted spatial distribution of the atoms inside the peptide molecule (Fig. 4B). It was observed that the radius of gyration of the peptide backbone was a function of time. In the simulation, the radius of gyration was found

to have oscillated to a greater degree before 20 ns, indicating that the structural convergence and folding occurred after 20 ns from the simulation being initiated.

Discussion

North-East India is endowed with diverse genetic resources of Bhut jolokia and wide variability exists in the plant and fruit characters (Islam et al. 2021). However, the productivity of the crop is mainly hindered by viral infections (Gutierrez Sanchez et al. 2020). A list of host genes associated with virus resistance has been documented in several previous plant-virus interaction studies (Diaz-Pendon et al. 2004; Gomez et al. 2009). However, it has been reported that there is an interaction between *eIF4E* and a potyvirus VPg (viral protein genome-linked), which leads to viral infectivity in plant species such as *Arabidopsis thaliana*, pea, pepper, potato and lettuce (Wittmann et al. 1997; Lellis et al. 2002; Kang et al. 2005; Duprat et al. 2002; Nicaise et al. 2003; Ruffel et al. 2005; and Lucioli et al. 2022). The abolishment of this interaction can lead to natural virus resistance in the plant (Kang et al. 2005; Lebedeva et al. 2024 and Leonard et al. 2000). The *eIF4E* and its isoform have been identified as vulnerability factors that are required for infection of potyviruses, cucumoviruses, bymoviruses and carmoviruses (Pyott et al. 2016; Yoshii et al. 2004; Stein et al. 2005; Nieto et al. 2006). In peanut (*Arachis hypogaea* L.), stripe virus infection requires translation initiation factors *eIF4E* and eIF(iso)4E (Xu et al. 2017). They isolated the full-length gene sequence using RACE primers and sequenced the Pea*eIF4E* and Pea *eIF(iso)4E* genes. The phylogenetic tree showed *eIF4E* and *eIF(iso)4E* in two distinct branches and pea*eIF4E* has clustered with the *eIF4E* subgroup, whereas pea *eIF(iso)4E* has clustered with the *eIF(iso)4E* subgroup. The cloned full-length sequence of lettuce *eIF4E* was of 1,032 nucleotides with a single open reading frame from position 21 to 710 and encoded a protein with a molecular mass of 26.1 Kd (Nicaise et al. 2006). The *eIF4E* cDNAs have closely matched with tomato, rice and Arabidopsis. An induced mutation of the *eIF4E* gene through CRISPR/Cas9 has improved the resistance against PVY in tobacco (Le et al. 2022). Brown et al. (2022) have isolated, cloned, and characterized the *eIF4E* protein genes (*eIF4E*, *eIFiso4E*and *CBP*) from four hexaploid sweet potato cultivars exhibiting susceptible/resistant and unknown phenotypic responses to sweet potato feathery mottle virus (SPFMV). Open reading frames were of 696 bp *IbeIF4E*, 606 bp *IbeIF(iso)4E*, and 675 bp *IbCBP* and the encoded single polypeptide lengths were 232, 202, and 225 amino acids for *IbeIF4E*, *IbeIF(iso)4E*, and *IbCBP* respectively. In the present study, Cc*eIF4E* has been observed to have ORF of 687 bp encoded 228 amino acid residues and substantially homologous to another eukaryotic translation initiation factor 4E from *C. annum* and *C. chinense*, with sequence identity of 95%. Multiple sequence alignment revealed that*CceIF4E* protein sequence is highly conserved with other known plant *eIF4E* protein sequences and contains the conserved amino acids at the N terminal regions. Dysfunctioning of the regulation of gene *eIF4E* can harm plant growth (Romagnoliet al. 2021). This structural information will help in designing *eIF4E*-targeted molecules, which can prevent viral infection by inhibiting the *eIF4E* from binding to VPg Bhut jolokia (*C. chinense* Jaqc.). This result will motivate the exploration of other possible options to prevent viral infections based on the molecular regulation of *eIF4E*.

This study successfully cloned and characterized the eIF4E gene from *C. chinense* Jacq, providing fundamental insights into its structure and function. The CceIF4E protein sequence was found to be highly conserved, with phylogenetic analysis indicating a close evolutionary relationship with *C. annum*. The results, including 3D modeling, offer valuable resources for future biotechnological improvements in enhancing disease resistance in this important crop.

Supplementary material

Supplementary Tables S1 and S2 are provided and can be accessed at www.isgpb.org

Authors' contributions

Conceptualization of research (RK, PS); Designing of the experiments (RK, PS); Contribution of experimental materials (RK, PS); Execution of field/lab experiments and data collection (SS, MD); Analysis of data and interpretation (RK, SS, MP, MD, PS); Preparation of the manuscript (RK, SS, MP).

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Supplementary Table S1. Designed degenerate primers

S. No.	Primer name	Primer length (nt)	Tm (°C)	GC %	Primer sequence	Expected product size (bp)
1	1F	22	51	40.9	5′-TGCTAATGAGGCAGATGATGAA-3′	247
	1R	22	53	45.4	5′-CTTGCTTGGGTGGTGGATATTA-3′	
2	2F	22	51	40.9	5′-TGATGAAGCTGAGAAGGTGAAA-3′	294
	2R	21	52	47.6	5′-AAGTCTGCTCCCACAACTAAC-3′	
3	3F	20	52	50.0	5′-CCAATGGAGGGACATGGAAA-3′	240
	3R	21	52	47.6	5′-CACTGTCGCTGTAATCCAGAA-3′	
4	4F	20	52	50.0	5′-CACCACCCAAGCAAGTTAGT-3′	371
	4R	20	52	50.0	5′-GCATTTCTGTCGAGCCTCTT-3′	

Footnote: List of the four pairs of degenerate primers designed from the highly conserved region of known *eIF4E*

Supplementary Table S2. List of designed gene specific primers

S. No.	Primer name	Primer length (nt)	Tm (°C)	GC %	Primer sequence	Expected product size (bp)
1	GSPF1	20	53	50.0	5′-TTTCCATGTCCCTCCATTGG-3′	400
	GSPR1	21	52	47.6	5′-GTGACAGCGACATTAGGTCTT-3′	
2	GSPF2	21	52	47.6	5′-GTTGTGGGAGCAGACTTACAT-3′	500
	GSPR2	22	53	45.4	5′-TCCCTTACCTCTGACACTAACT-3′	

Footnote: List of the two pairs of gene specific primers, designed based on sequence of the product amplified by using degenerate primer pair 4