

Genetic profiling of eukaryotic Translation Initiation Factor 4E (eIF4E) in Cabai Berangkai variant (*Capsicum Annuum*): Comparative Alignment and Exon Sequencing Approach

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Abstract

Eukaryotic Translation Initiation Factor 4E (eIF4E) has been demonstrated as a key regulator of protein translation in vivo and it participates in plant defense mechanisms against viral pathogens. In this study, we examined the genetic variation within a local pepper variant (*Capsicum annuum*) called Cabai Berangkai (CB), which is characterized by the eIF4E gene. DNA isolation and sequencing of the eIF4E gene were performed, followed by comparative analysis with four reference cultivars: Doux Long Des, Landes (DDL), Yolo Wonder (YW), FloridaVR2 (F), Yolo Y (YY). We detected significant allelic variation in the eIF4E gene of CB with unique single nucleotide polymorphisms (SNPs). Results showed that comparison of the amino acids sequence of the eIF4E gene indicates high allele variation with the reference cultivars. CB also showed a unique amino acid substitution in the eIF4E protein among the different strains, possibly indicating genetic differentiation. These findings aid in understanding pepper genetics and are particularly relevant to the potential exploitation of diversity of eIF4E through breeding programs seeking increased viral resistance.

Key words: *Capsicum annuum*, Eukaryotic Translation Initiation Factor 4E (eIF4E), SNPs, viral resistance

Introduction

Plant genetics research enhances agricultural resilience and productivity, particularly for economically significant crops like chili (*Capsicum annuum*). Chili is an important part of Indonesian cuisine, making it an important national commodity. Its value is highlighted by its broad usage in various traditional foods. Over the past several years, Indonesia's chili production has increased significantly, suggesting that the nation has the potential to become a major producer worldwide. Indonesia's output increased by 4.76% between 2018 and 2020 and a more significant 14.26% between 2021 and 2023. As a consequence, throughout the five years from 2018 to 2023, chili output increased by a total of 28.83% (BPS, 2023).

The Cabai Berangkai chili cultivar is commonly distributed and popular among Indonesian farmers due to its capacity to produce more fruits per branch than other chili varieties. However, it has been noted that Cabai Berangkai is more vulnerable to viral infections than other chili varieties. This increased susceptibility is a substantial problem for farmers since viral infections can result in severe output and quality losses. Viral infections are a significant risk to chili farmers, as they have been able to spread quickly and cause crop loss of up to 100%. This problem gets worse because of Indonesia's fluctuating climate and the virus's capacity to spread to other locations. Pesticides and other methods have been used to control whiteflies, which are the main vectors of these viruses, yet they have proven ineffective in preventing disease transmission.

Viruses, made entirely of DNA or RNA, lack their own transcription and translation machinery and instead rely on "hijacking" the host's biological components to replicate (Kumar

2019). This fundamental vulnerability provides an opportunity to investigate genetic-based methods of managing viral infections in chili crops.

Various research investigations into the interactions between plants and viruses have uncovered complex systems that influence both the immune responses of plants and the pathogenicity of viruses. The process through which RNA viruses infect chili plants has been the subject of considerable research, showing a significant interaction with the eukaryotic translation initiation factor 4E (eIF4E) complex (Ruffel *et al.*, 2002; Ruffel *et al.*, 2004) conferring recessive resistance against strains of potato virus Y (PVY). eIF4E is an essential element of host translation machinery because it binds to the mRNA 5'-cap, translating host proteins efficiently. For RNA viruses, eIF4E is required for viral protein expression, allowing the replication of viruses and their spread throughout the plant.

Additionally, mutations in the eIF4E gene have been demonstrated to affect its interaction with viral proteins, including the viral genome-linked protein (VPg), which is required for potyvirus replication (Kan *et al.*, 2023). These alterations pretty much halt viral growth and offer fairly robust resistance. These findings emphasize the importance of eIF4E in determining plant susceptibility or resistance to RNA viruses. Furthermore, eIF4E-mediated resistance has been extensively documented across multiple crop species like tomato (Yoon *et al.*, 2020) including eIF4E, eIF4G, and related proteins. Notably, eIF4E and its isoform eIF(iso) have been studied across several crops for their role in virus interactions. In cassava (Gomez *et al.*, 2019), they are associated with susceptibility to the genus Ipomovirus, particularly Cassava brown streak virus (CBSV). In tobacco (Le *et*

al., 2022), infection by potyviruses such as Potato virus Y (PVY) can reduce yield by up to 70%. Similarly, in potato (Gutierrez Sanchez *et al.*, 2020), these factors are frequently linked to multiple minor effects on plant growth and overall production.

Identifying key genes that influence this variety and investigating potential genetic alterations may improve its resilience, resulting in increased yields and stability for farmers who rely on this critical crop. Here we compare the eIF4E gene in Cabai Berangkai to other cultivars known to be susceptible or resistant to RNA viruses. The findings of this investigation will help determine whether Cabai Berangkai is vulnerable or resistant to RNA viruses.

Materials and methods

Plant materials: Cabai Berangkai pepper plants were successfully regenerated from callus cultures using explants taken from healthy specimens. The regeneration process began with carefully selecting explants cultured in a controlled environment to promote callus formation. Once the callus cultures were established, the regenerated plants were nurtured under controlled conditions to ensure optimal growth. After reaching sufficient maturity, leaves were harvested for DNA isolation.

DNA isolation: Genomic DNA was extracted from fresh leaves, which were cut into small pieces approximately 1 cm in size, using the GeneJET Genomic DNA Purification Kit. Fresh leaves were collected and placed in a microcentrifuge tube, to which 200 μ L of Lysis Solution was added. The mixture was thoroughly vortexed for 15 seconds to ensure homogeneity. Following this, 400 μ L of 50% ethanol was added to the lysate, and the sample was mixed gently by pipetting. The prepared lysate was then transferred to a GeneJET Genomic DNA Purification Column positioned in a collection tube and centrifuged at $6000 \times g$ for 1 minute. The flow-through solution was discarded, and the column was placed into a new collection tube. Subsequently, 500 μ L of Wash Buffer I (supplemented with ethanol as per the manufacturer's instructions) was added to the purification column, which was then centrifuged at $8000 \times g$ for 1 minute. After discarding the flow-through, the column was returned to the collection tube. Next, 500 μ L of Wash Buffer II (also supplemented with ethanol) was added to the purification column, and it was centrifuged at maximum speed ($\geq 12000 \times g$) for 3 minutes. If any residual wash buffer remained, a re-spin for an additional minute was performed. The GeneJET purification column was then transferred to a clean 1.5 mL microcentrifuge tube. To elute the genomic DNA, 200 μ L of Elution Buffer was added to the center of the column membrane and incubated for 2 minutes at room temperature before centrifuging at $8000 \times g$ for 1 minute. For optimal yield, this elution step was repeated with an additional 200 μ L of Elution Buffer. Finally, the purification column was discarded, and the purified genomic DNA was stored at $-20 \text{ }^\circ\text{C}$ for future use.

Primer design and PCR amplification: Specific primers targeting the entire exon region of the eIF4E gene were designed based on conserved sequences from related pepper species. The primer sequences and PCR conditions are detailed in Table 1. The PCR cycling parameters for amplifying the eIF4E gene using the specified primers were established as follows: an initial denaturation step was performed at $95 \text{ }^\circ\text{C}$ for 5 minutes, followed by 30 cycles of denaturation at $95 \text{ }^\circ\text{C}$ for 30 seconds, annealing

at varying temperatures depending on the primer used for 30 seconds, extension at $72 \text{ }^\circ\text{C}$ for 1 minute and final extension at $72 \text{ }^\circ\text{C}$ for 5 minutes.

Electrophoresis: The PCR products were visualized on a 0.8% ethidium bromide-stained agarose gel with a 1kb DNA standard in 0.5x TBE buffer. The gel was then run at a constant voltage of 80 V for 60 minutes until the dye front had migrated an adequate distance, and the gel was placed on the gel documentation system to visualize the DNA bands.

Table 1. Primer name and sequence of eIF4E region

Primer Name	Sequence (5' to 3')	Annealing Temperature ($^\circ\text{C}$)	PCR product size (bp)
eIF4E01	GGC ATT TAA CAA AGA ATC TCA CCG CAA CCC ACG CCT TGT TCG TGA TTG TTC GAT TCC CCT AAT ACC C	64	540
eIF4E02	CAG GCC AGT ATT TTC AGT TTC ATG CAT GGA CCT T CAA TAA AGT TGG ACA AAC ACA GAG TGA GGC GAA G	64	946
eIF4E03	GAC GAG ACA TCT AGA TAT CCC TGT GAG AGG TGA T GGC ATG AAT CCA AAA CCG GAC AAT TTT ACG CGA	64	720

DNA sequencing and sequence analysis: After amplification, the PCR products were sent for Sanger sequencing at the 1st BASE DNA sequencing facility. The sequencing results were analyzed using BioEdit sequence alignment editor version 7.0.5.3. This software facilitated confirmation of the identity of the amplified fragments by aligning the obtained sequences with reference sequences.

Comparative analysis: The obtained eIF4E sequences from Cabai Berangkai were compared with the eIF4E sequences of four reference pepper cultivars: Yolo Wonder (AY122052.1), Doux Long des Landes (AF521963.1), Yolo Y (AF521964.1), and FloridaVR2 (AF521965.1), which were retrieved from NCBI public databases. To identify any sequence variations or conserved regions, multiple sequence alignment was performed using BioEdit sequence alignment editor version 7.0.5.3.

Result and discussion

PCR amplification: The electrophoresis results using primers eIF4E01, eIF4E02, and eIF4E03 showed a single clean band with the expected size, namely bands with sizes of 540 bp, 946 bp, and 720 bp representing exon 1, exon 2-3, and exon 4-5, respectively (Fig. 1.). These bands indicate that PCR amplification using primers eIF4E01, eIF4E02, and eIF4E03 is specific and effective amplify the target region of the eIF4E gene. Visualization of these products is critical to validate the integrity of the PCR product and ensure that subsequent analysis can be performed accurately.

DNA sequencing and sequence analysis: DNA sequencing of the PCR products yielded 3 partial sequences corresponding to the four exons of the eIF4E gene in the Chili Berangkai chili plant. To reconstruct the sequence of a single eIF4E gene intact, contig assembly is performed using alignment software. This alignment method combines overlapping sequences from multiple fragments to create a single sequence, known as a contig. The alignment results were compared with *Capsicum Annum* mRNA transcripts

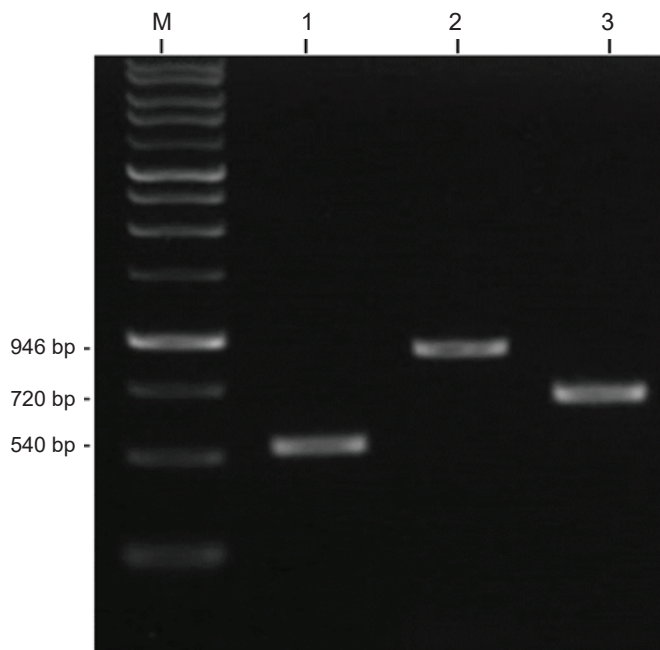


Fig. 1. Agarose gel electrophoresis of PCR products amplifying different exons of the eIF4E gene in Cabai Berangkai pepper plants. Lane M: 1 kb DNA ladder; Lane 1: exon 1; Lane 2: exons 2-3; Lane 3: exons 4-5. The results reveal clean, distinct bands corresponding to the expected sizes of approximately 540 bp for exon 1, 946 bp for exons 2-3, and 720 bp for exons 4-5.

(NM_001324970.1) and genome sequences (NC_061114.1) retrieved from NCBI, resulting in the reconstruction of a single intact eIF4E gene sequence.

CB eIF4E gene is 687 bp in size and consists of five exons with varying sizes, namely 278 bp, 166 bp, 126 bp, 66 bp, and 51 bp. These five exons were analyzed to determine the variation in CB eIF4E gene. The eIF4E gene in the genotypes Yolo Wonder (YW), Doux Des Landes (DDL), Yolo Y (YY), and FloridaVR2 (F) also has an exon size of 687 bp showing no significant variation in exon size between CB and other genotypes.

Comparative analysis: A comparison of the amino acid sequence between CB and the reference genotype showed a similarity of 99.12%. Further examination shows CB has four amino acid substitutions (Fig. 2a). These mutations are known to have no implications on mRNA 5' activity and eIF4G complex formation.

CB genotype has Glu at position 67, which is similar to both resistant genotypes YY and F, but different from susceptible genotypes YW and DDL. However, the CB genotype has Arg at position 79, replacing Leu, which is not similar to the resistant genotype YY. Then Asp at position 109, replacing Asn, which is different from the resistant genotype F. Interestingly, the CB genotype has Gly replacing Asp at position 205, a substitution not found in either the resistant or susceptible genotypes. This amino acid substitution occurs due to a nucleotide change from adenine (A) to guanine (G) at position 614 in the nucleotide sequence (Fig. 2b).

The two resistant genotypes, Yolo Y and Florida VR2, have the same amino acid substitution, Glu replacing valine Val at position 67. However, the two resistant genotypes have one amino acid difference. Yolo Y has an Arg substitution for Leu at position 79, while Florida VR2 has an Asn substitution for Asp at position 109. In contrast, the susceptible genotypes, Yolo Wonder (YW) and

Doux Long des Landes (DDL), show 100% amino acid sequence concordance.

The findings in this study provide critical insights into the functional consequences of amino acid substitutions in the eIF4E gene of the Chili Berangkai (CB) genotype and their implications for viral resistance. The CB eIF4E gene exhibited four amino acid substitutions when compared to reference genotypes, which include both resistant and susceptible varieties. These mutations—Glu at position 67, Arg at position 79, Asp at position 109, and Gly at position 205—are particularly significant when analyzed in the context of viral susceptibility and resistance mechanisms.

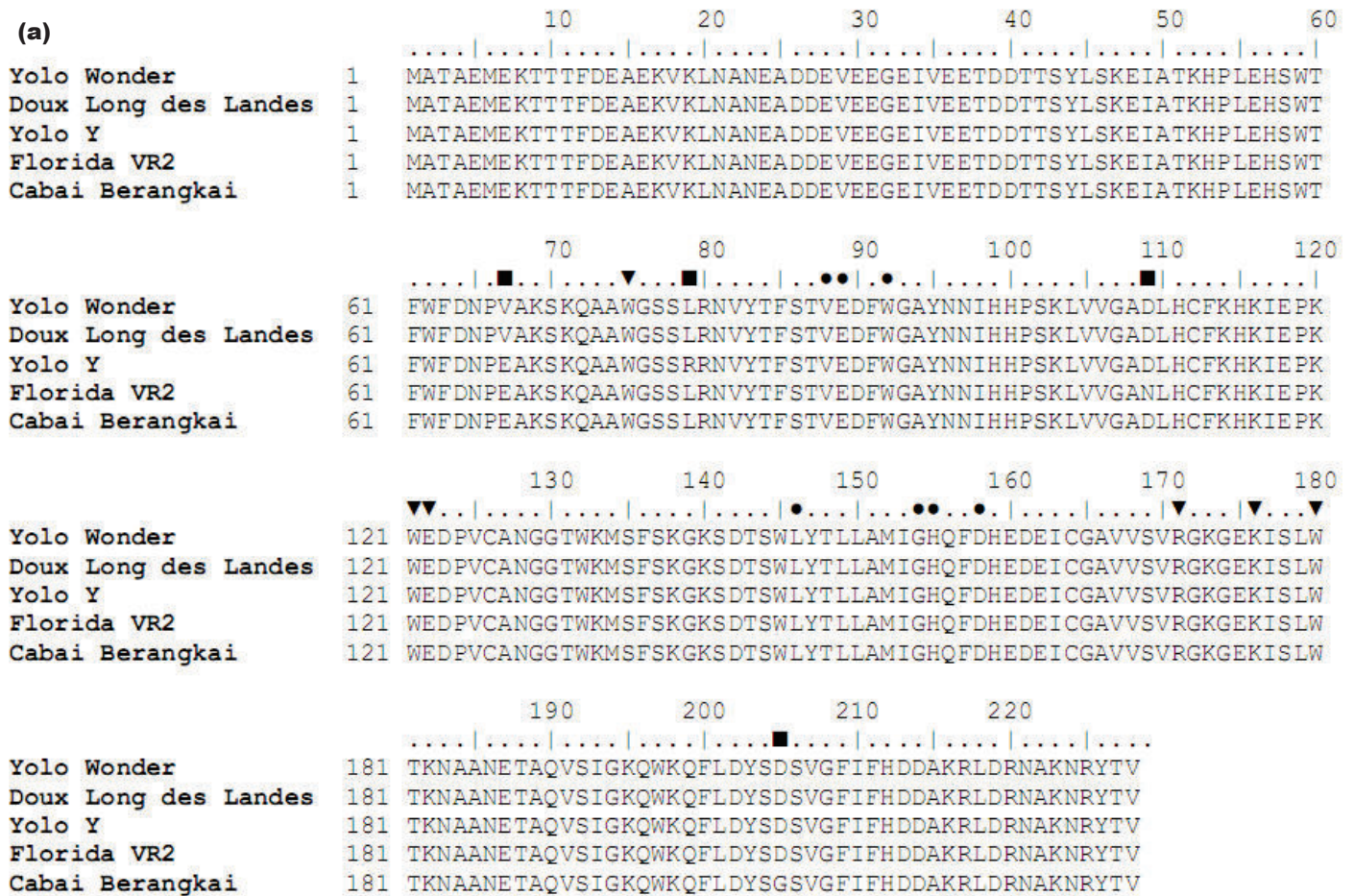
Functional consequences of amino acid substitutions: The substitution of Glu at position 67 aligns the CB genotype with resistant genotypes Yolo Y (YY) and Florida VR2 (F), as opposed to susceptible genotypes Yolo Wonder (YW) and Doux Des Landes (DDL), which retain Val at this position. This mutation is critical because previous studies have demonstrated that amino acid changes in eIF4E can disrupt its interaction with viral proteins such as VPg. VPg is a key factor used by potyviruses to hijack the host's translation machinery for viral replication (Yeam *et al.*, 2007; Zlobin and Taranov, 2023), highlighting the potential structural roles of these mutations. The presence of Glu at position 67 may contribute to a loss-of-susceptibility mechanism, rendering CB partially resistant to certain viruses.

However, the Arg substitution at position 79 and the Asp substitution at position 109 distinguish CB from the resistant genotypes YY and F. These changes may change the structural conformation of eIF4E, altering its capacity to bind VPg or other host components essential for viral replication. Remarkably, Gly replacing Asp at position 205 is unique to CB and does not occur in susceptible or resistant genotypes. This alteration may significantly affect how eIF4E interacts with host and viral proteins. It entails a nucleotide change from adenine (A) to guanine (G) at position 614.

The findings of this study are consistent with previous research demonstrating that point mutations in eIF4E can confer resistance by disrupting its interaction with viral proteins. For example, studies on *Capsicum* species have shown that non-conservative amino acid changes in eIF4E, such as G107R, interrupt VPg binding and cap-binding ability, thereby impeding viral replication (Yeam *et al.*, 2007) the potential structural role(s). Similarly, mutations in eIF4E isoforms have been associated with recessive resistance to potyviruses across various plant species (Zlobin and Taranov, 2023; Yoon *et al.*, 2020). The CB genotype's unique Gly205 substitution is particularly intriguing because it represents a novel mutation not previously reported in other genotypes.

While the CB genotype shares some similarities with resistant varieties like YY and F, it also exhibits distinct differences that may influence its resistance spectrum. The partial sequence similarity (99.12%) between CB and reference genotypes highlights the high conservation of eIF4E but also underscores the functional significance of even minor amino acid changes (Yeam *et al.*, 2007) the potential structural role(s).

Implications for viral resistance: The mutations found in CB's eIF4E gene are likely to have an influence on its function as a viral susceptibility factor. Potyviruses require eIF4E to bind to their VPg protein46 to commence translation and spread throughout



(b) **TTACAGCGG CAGTGTTGGCT**

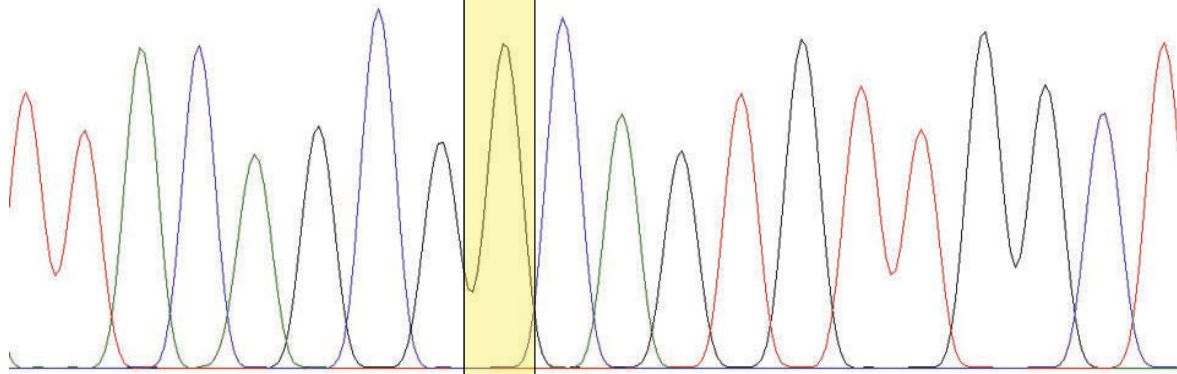


Fig. 2. (a) Alignment of Cabai Berangkai eIF4E amino acid sequences with those of Yolo Wonder, Doux Des Landes, Yolo Y, and FloridaVR2. Amino acids involved in binding the mRNA cap structure are marked with black circles, while those involved in recognizing eIF4G and 4E-binding proteins are indicated by arrowheads. Differences between Cabai Berangkai and reference sequences are highlighted with open boxes. (b) Amino acid substitution in the CB genotype at residue 205, where aspartic acid (Asp) is replaced by glycine (Gly) due to a nucleotide change from adenine (A) to guanine (G) at position 614 in the nucleotide sequence

the host. Amino acid alterations that impair this connection may limit viral reproduction and spread. For instance, Glu67 may enhance resistance by mimicking mutations found in other resistant genotypes. However, the unique Gly205 substitution could introduce novel structural or functional changes that either enhance or diminish resistance depending on the specific virus strain.

The combination of mutations in the CB genotype does not seem to affect important eIF4E activities linked to cellular mRNA translation, since no developmental problems have been reported.

The findings indicate that some eIF4E mutations can confer viral resistance without adversely affecting plant health (Sanfaçon, 2015; Yoon *et al.*, 2020). The study's results align with broader evidence suggesting that natural variation in eIF4E plays a pivotal role in plant-virus interactions. Recessive resistance mediated by eIF4E is frequently observed in domesticated crops due to its mild pleiotropic effects on growth and productivity. However, because viruses may overcome single-point alterations in eIF4E (Montero *et al.*, 2015; Yeam *et al.*, 2007), their resistance-breaking mutations remain a concern. Targeting different

susceptibility factors or undergoing many mutations may be necessary to attain long-term resistance.

This work emphasises the importance of amino acid changes in the eIF4E gene of the Chili Berangkai (CB) genotype in giving resistance to viral infections. The discovery of four critical mutations—Glu67, Arg79, Asp109, and Gly205—provides vital information on the molecular processes behind viral resistance. Notably, the Glu67 substitution aligns CB with resistant genotypes, while the unique Gly205 substitution introduces a novel variation that may influence resistance specificity. These findings have crucial implications for breeding programs because they suggest that particular eIF4E modifications can be employed to boost pepper cultivars' viral resistance without compromising plant output or growth. To translate these findings into practical agricultural applications, future research should focus on conducting field trials to evaluate the performance of CB and other genotypes under natural viral infection conditions. Additionally, the functional effects of the unique Gly205 substitution should be further investigated to determine its impact on resistance against diverse potyvirus strains. Extending this study to other pepper cultivars and comparable crops might provide a more thorough understanding of eIF4E-mediated resistance mechanisms as well as support in the development of strong, multi-virus-resistant cultivars. It may be possible to improve crop resilience and ensure sustainable agriculture practices by adding molecular insights into breeding processes.

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