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AP2/ERF DOMAIN CONSERVATION IN *PARTHENIUM HYSTEROPHORUS*: A B-SHEET-DRIVEN *DREB1/CBF* HOMOLOG UNVEILED FOR FROST ADAPTATION AND INVASION SUCCESS

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Abstract

The invasive weed *Parthenium hysterophorus* exhibits remarkable adaptability to abiotic stresses, yet the molecular mechanisms underpinning its cold tolerance remain unexplored. This study investigates the role of the *DREB1/CBF* transcription factor in mediating low-temperature resilience in *P. hysterophorus* and functional analysis of the *DREB1/CBF* gene. Physiological and molecular analyses were performed on plants exposed to cold (4°C) and control (25°C) conditions. Cold-acclimated plants (4°C) exhibited enhanced membrane stability compared to non-acclimated control plants, Carotenoids contents increased 19.9% in cold acclimated plants compared to control. A notable decrease found in chlorophyll a (2.78 µg/g) and chlorophyll b (1.26 µg/g) in cold-treated plants compared to controls (3.14 µg/g and 1.62 µg/g, respectively). Molecular analysis identified a first ever novel *DREB1/CBF* homolog in *P. hysterophorus*, showing significant up-regulation under cold stress via PCR amplification. Bioinformatics revealed >90% nucleotide sequence homology with *Brassica* species, with conserved AP2/ERF DNA-binding domains critical for CRT/DRE motif recognition. Structural modeling predicted a stable globular protein with solvent-exposed β-sheets, validated through refinement (ERRAT: 95.6%; Ramachandran favored regions: 93.3%). Phylogenetic clustering with *Brassica napus* and *B. oleracea* underscores evolutionary conservation of cold-response pathways. These findings provide novel insights into the molecular basis of invasiveness in *P. hysterophorus*, highlighting dual applications for targeted weed management and the development of climate-resilient crops through *DREB1/CBF* engineering.

Key words: *Parthenium hysterophorus;* Low temperature stress; *DREB1/CBF* gene; BLAST

Introduction

Parthenium hysterophorus, commonly known as Parthenium weed is a member of the Asteraceae family. An annual herbaceous plant with height varies from 1-1.5 or (-2) meters. The leaves are divided deeply, and appear like ferns. They have capitula which are white in color, small in size and are clustered in groups at the ends of branches (Adkins & Shabbir, 2014). It is a highly invasive noxious weed that spreads aggressively across farmlands, roadsides, and natural ecosystems (Hadi *et al.,* 2014). Its ability to grow rapidly and produce thousands of cypselas allows it to overpower native plants, reduce crop yields by up to 50%, and harm livestock health through toxic chemicals (Khan *et al.,* 2012; Bashar *et al*., 2021). Along with its destructive nature, this weed survives extreme environmental conditions, including freezing temperatures that would not support most plants. Understanding how it adapts to such stress could reveal strategies to control its spread or even improve cold tolerance in crops. Cold stress damages plants by breaking down cell membranes, reducing photosynthesis, and generating harmful reactive oxygen molecules (Gilmour *et al*., 2004; Pradhan *et al.,* 2019). To survive, plants activate genes like *DREB1/CBF*, which act as molecular switches to turn on protective mechanisms (Nasir & Hadi, 2018). These genes help plants produce antioxidants, stabilize cell structures, and repair damage caused by low temperatures (Zhang & Xia, 2023). The role of *DREB1/CBF* is well-studied in crops like wheat and rice, its role in invasive weeds like *Parthenium hysterophorus* remains unknown. Current study investigates how *Parthenium hysterophorus* survives frost by analyzing the *DREB1/CBF* gene. We tested two hypotheses: (1) Cold acclimation increases the expression of the *DREB1/CBF* gene in *Parthenium hysterophorus* weed. (2) The gene's structure and function are similar to known cold-tolerance genes in related plants. We measured changes in plant physiology (e.g., chlorophyll levels, cell membrane damage) under cold conditions and compared the *DREB1/CBF* gene sequence to those of other species. Our results suggest that this gene plays a critical role in the parthenium weed's frost resistance. These findings could help in future weed management and for further molecular and biochemical investigations of invasive species or using its genes to develop stress resilience in crops through genetic engineering and biotechnology. The current study was carried out with objectives. (1) To identify *DREB1/CBF* gene in *P. hysterophorus*. (2) To find out the effect of cold acclimation on the expression of *DREB1/CBF* gene and on photosynthetic pigment in *P. hysterophorus* (3) to compare the gene sequence with other plants genes using bioinformatics tools.

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Materials and Methods

Plant material and experimental design: Mature cypselas of *P. hysterophorus* were collected from agricultural fields and roadsides in vicinity of the University of Malakand, Pakistan. After surface sterilization, the seeds were sown in pots containing 148 g of soil (pH 6.8) collected from the university's botanical garden. The plants were grown in the Molecular Stress Physiology Laboratory under controlled conditions (25°C with a 16/8-hour light/dark cycle) for two months. Following the growth period, the plants were split into two groups: a control group maintained at 25°C (ambient temperature) and a treatment group exposed to cold stress at 4°C for 24 hour.

Physiological Assessments

Electrolyte leakage assay: Leaf discs (10 mm in diameter) were excised from both control and cold-treated plants and subjected to freeze-thaw cycles. Relative electrical conductivity (REC %)—an index of membrane integrity—was calculated using conductivity measurements taken before and after autoclaving the samples. The REC% was computed using the equation:

$$REC\% = \text{Post freezing EC}/\text{Post autoclaving EC} \times 100$$

This method adapted from Gilmour *et al*., (2004) provided a quantitative assessment of cell membrane stability under cold stress.

Biochemical profiling for pigment quantification: Pigments were extracted from 0.1 g of fresh leaf tissue using 80% (v/v) acetone. Absorbance readings were taken at 663 nm, 645 nm, and 470 nm with a UV–Vis spectrophotometer (Shimadzu UV-1800). Chlorophyll a, chlorophyll b, and carotenoid concentrations were calculated with the following equations (Arnon 1949),

- Chlorophyll a (µg/mL) = $12.7 \times A663 - 2.69 \times A645$
- Chlorophyll b (µg/mL) = $22.9 \times A645 - 4.68 \times A663$
- Carotenoids (µg/mL) = $(1000 \times A470 - 1.82 \times \text{Chl a} - 85.02 \times \text{Chl b}) / 198$

RNA extraction*:* Total RNA was extracted from leaves of both cold-acclimated and non-acclimated plants. To prevent RNA degradation, tissue samples were immediately flash-frozen in liquid nitrogen. All surfaces and equipments were pre-treated with an RNase decontamination solution (Sigma RNase ZAP, Cat # R2020). The frozen tissues were pulverized in liquid nitrogen, and approximately 100 mg of the resulting powder was transferred into RNase-free microcentrifuge tubes (1.5 mL, Eppendorf) for RNA extraction (Hadi *et al*., 2011).

cDNA synthesis and PCR amplification: First-strand cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, Cat # A3800) according to the manufacturer's instructions. In each reaction, 0.8 µg of RNA was diluted with nuclease-free water (Sigma, Cat # W1754) and combined with oligo (dT) 15 primers in a nuclease-free PCR tube (0.2 mL, Ambion) to a total volume of 5 µL. The reverse transcription was performed in a Perkin Elmer 9700 thermal cycler with an incubation at 25°C for 5 minutes (annealing), 42°C for 60 minutes (extension), and 70°C for 15 minutes (enzyme inactivation). PCR amplification of the DREB1/CBF gene was conducted in a 50 µL reaction mixture containing 25 µL of 2× PCR Master Mix (Promega, Cat # M7502), 5 µL of each primer (10 µM), 5 µL of cDNA, and nuclease-free water to bring the total volume to 50 µL. The degenerate primers used were:

- Forward: 5'-AAGAAGTTTCGTGAGACCCGTCAC-3'
- Reverse: 5'-GGCAAAAGCATACCTTCCGCCAT-3'

The thermal cycling protocol consisted of an initial denaturation at 94°C for 3 minutes; 35 cycles of 94°C for 1 minute, 61°C for 1 minute, and 72°C for 2 minutes; followed by a final extension at 72°C for 10 minutes. PCR products were separated on a 0.8% agarose gel (Sigma) and visualized using UV trans illumination. Band sizes were compared with a PCR marker ladder (Promega, Cat # G3161) and quantified using Quantity One 4.6.3 Bio-Rad software (Hadi *et al*., 2011).

Gene sequence retrieval and bioinformatics analysis: The amplified CBF/DREB1 gene fragment was converted into FASTA format and subjected to BLASTn analysis to identify homologous sequences from public databases. Additionally, BLASTx was performed to locate related protein sequences (Ren *et al*., 2021). The four best-matching sequences—selected based on percent identity, E-value, and alignment score—were downloaded and aligned using ClustalW2 (EMBL-EBI) to detect conserved motifs and sequence variations (Fuller *et al*., 2017).

Protein translation and structural analysis: The nucleotide sequence of the CBF/DREB1 gene was converted into its amino acid sequence using the Expasy Translate Tool (Javan & Alipanah, 2022). To identify structural features such as α-helices, β-sheets, and loops, the resulting protein sequence was analyzed with PSIPRED, a tool that predicts secondary structures from amino acid data (Buchan & Jones, 2019). For 3D modeling, the protein sequence was subjected to trRosetta, a computational platform that uses deep learning algorithms to estimate spatial relationships between amino acid residues and construct structural models. These preliminary models were subjected to quality checks using ERRAT (for atomic interactions), Verify3D (sequence-structure compatibility), and Procheck (backbone conformation). To enhance accuracy, the best-performing model was refined with GalaxyRefine (Galaxy Web), which optimizes side-chain positioning and overall geometry (Ko *et al.,* 2012). The final refined structure was selected for downstream functional studies.

Data analysis

Electrolyte leakage (EC) values were statistically compared using Microsoft Excel. Gene sequence similarities were identified through BLAST searches, and multiple sequence alignments were performed with ClustalW2 to detect conserved regions. Protein secondary structure predictions from PSIPRED were cross-validated with the 3D models generated by trRosetta. Structural refinements via GalaxyRefine were verified through ERRAT, Verify3D, and Procheck to ensure improved reliability.

Results

Expression analysis of *DREB1/CBF* gene in *Parthenium hysterophorus* under low-temperature stress: The *DREB1/CBF* gene was successfully identified in *P. hysterophorus* for the first time. PCR amplification confirmed the isolation of an exon-containing sequence. Differential expression was observed under cold acclimation (4°C) versus room temperature (25°C). At 25°C (3 replicates bands) showed weaker band intensity indicated basal transcription levels, while dense bands at 4°C treatment, significant up-regulation of *DREB1/CBF* (Fig. 1). This suggests enhanced transcriptional activity of the gene under cold stress, consistent with its role as a key regulator of low-temperature responses.

Freezing tolerance and membrane stability: Electrolyte leakage assays revealed contrasting effects of temperature treatments on membrane integrity. Non-acclimated plants (25°C) exhibited a high relative electrical conductivity (REC%) of 48%, indicative of membrane damage. In contrast, cold-acclimated plants (4°C) showed a markedly reduced REC% of 21% (Fig. 2), signifying improved membrane stability. This reduction aligns with the observed up-regulation of *DREB1/CBF*, which likely activates downstream cold-responsive (*COR*) genes to fortify cellular structures against freezing-induced damage.

Chlorophyll and carotenoid dynamics under cold stress: Biochemical profiling of photosynthetic pigments revealed temperature-dependent variations (Fig. 3). Chlorophyll *a* was decreased from 3.14 µg/g (25°C) to 2.78 µg/g (4°C), while chlorophyll *b* was declined from 1.62 µg/g to 1.26 µg/g. In contrast, carotenoid content was significantly increased by 19.9% under cold acclimation (1.96 µg/g to 2.35 µg/g), suggesting a compensatory antioxidant mechanism to mitigate oxidative stress during low-temperature adaptation.

Sequence homology and phylogenetic relationships: BLASTn analysis of the *DREB1/CBF* cDNA sequence (accession: AN335566) revealed high similarity (>90% identity) to homologs in *Brassica* species (Table 1). Form *Brassica napus*, *BnDREB1B* and *BnCBF5* are different entries, however, the *DREB1/CBF* gene family is highly conserved across different species, so, they exhibit the same identity score to PhCBF/DREB1. Multiple sequence alignment (MSA) highlighted conserved regions across taxa (Fig. 4A–B), including the AP2/ERF DNA-binding domain. Phylogenetic reconstruction using the Neighbor-Joining method grouped *P. hysterophorus* within a clade containing *Brassica napus* (AF499031) and *Brassica oleracea* (XM013770359), indicating evolutionary conservation of cold-response pathways (Fig. 4C).

Protein structure prediction and validation: Translation of the nucleotide sequence yielded a 60-amino-acid polypeptide. PSIPRED analysis predicted secondary structural elements, including α-helices (residues 52–55) and β-sheets (residues 4–6, 19–21, 24–33, 40–46, and 48–51), critical for protein stability (Fig. 5A–B). The 3D model, generated using trRosetta (TM-score: 0.440), was refined via GalaxyRefine, improving validation metrics:

- **ERRAT**: Overall quality score increased from 90 to 95.6% (Fig. 6A–B).
- **Ramachandran plot:** 93.3% of residues occupied favored regions post-refinement (Fig. 7A–B).
- **Verify3D:** Sequence-structure compatibility rose to 80%, confirming model reliability (Fig. 8A–B).
- **The preliminary 3D model:** Generated by trRosetta (Fig. 9A) shows basic folding with several regions exhibiting unfavorable atomic interactions while the GalaxyRefine-optimized structure with improved compactness and enhanced validation (93.3% favored, ERRAT 95.6%, Verify3D 80%), notably within the AP2/ERF domain (Fig. 9B).

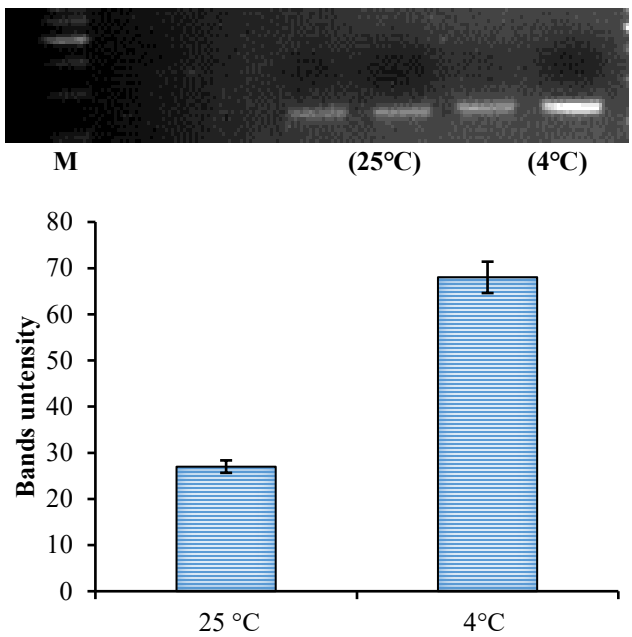

Fig. 1. *DREB1/CBF* expression in *P. hysterophorus* under various conditions of temperature. M shows the molecular marker. At 25°C (room temperature) three replicates bands exhibit lower bands intensity representing lower expression of the gene when compared with low temperature (4°C) treatment, indicates a more intense band, showing higher expression of transcript under low temperature.

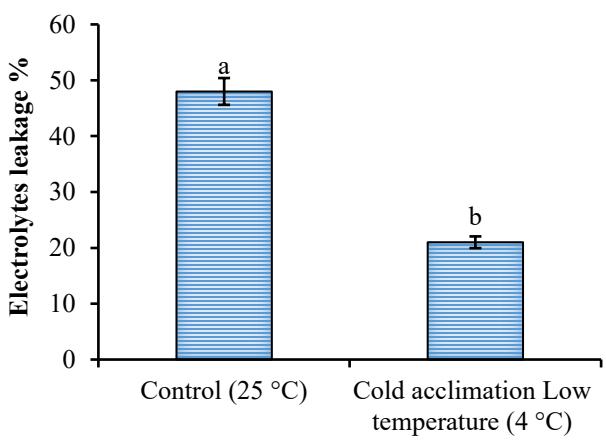

Fig. 2. Relative electrical conductivity REC% of acclimated (4°C) and non-acclimated (25°C) treated plants leaves.

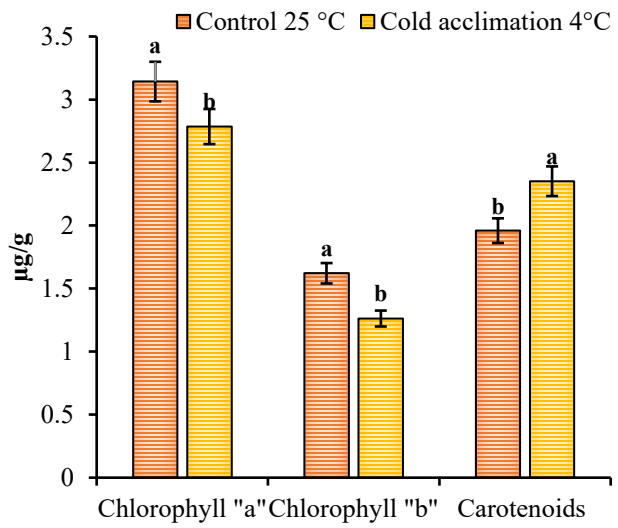

Fig. 3. Chlorophyll and carotenoids contents in leaves of control and cold acclimated plants.

Functional implications of structural features: The refined model revealed a compact globular domain with solvent-exposed β-sheets, potentially facilitating DNA binding. The conserved AP2/ERF motif (residues 14–25) aligns with known *DREB1/CBF* proteins, supporting its role in regulating cold-responsive gene promoters.

Discussion

This study provides critical insights into the molecular mechanisms enabling *P. hysterophorus* to survive frost stress, highlighting the pivotal role of the *DREB1/CBF* gene. The observed up-regulation of *DREB1/CBF* under cold conditions aligns with its known function in activating protective pathways, such as stabilizing cell membranes and reducing oxidative damage (Zhang & Xia, 2023). In *P. Hysterophorus*, *DREB1/CBF*'s cold induced up regulation shows its role in adaptation of stress. Similar results have also been reported in other invasive species like *Ambrosia artemisiifolia* (Battlay *et al*., 2023). Other recent studies of multi omics (Chen *et al*., 2024), reported that weeds could rapidly adapt their activity of genes to grow and persist new conditions of environment, supporting our findings. The significant reduction in electrolyte leakage in cold-acclimated plants supports the idea that *DREB1/CBF* enhances membrane integrity, likely by triggering the production of cryoprotective compounds. These findings mirror patterns seen in cold-tolerant crops like *Arabidopsis* and *Brassica*, suggesting conserved stress-response mechanisms across diverse plant species (Gilmour *et al.,* 2004; Li *et al.,* 2020). Another study highlights that under cold acclimation, the reduction in electrolyte leakage supports *DREB1/CBF* role to protect membranes, reliable with new studies showing *DREB1/CBF* as cold stress core regulators (Peng *et al*., 2025).

The decline in chlorophyll levels (chlorophyll *a* by 11.5%, chlorophyll *b* by 22.2%) under cold stress indicates photosynthetic disruption, a common consequence of low temperatures (Hadi *et al.,* 2011). However, the simultaneous 19.9% increase in carotenoids suggests a compensatory antioxidant mechanism to mitigate reactive oxygen species (ROS) generated during stress (Nasir *et al.*, 2019). Cold stress frequently decreases levels of chlorophyll, as shown in recent reviews, however, our study demonstrates an adaptive increase in carotenoids which can help balance oxidative damage (Feng *et al.*, 2025). This balance between chlorophyll loss and carotenoid gain reflects an adaptive strategy to prioritize survival over growth under harsh conditions, a trait that may contribute to *P. hysterophorus*'s invasiveness.

The discovery of a *DREB1/CBF* homolog with >90% sequence similarity to *Brassica* species underscores evolutionary conservation in cold-response pathways. The conserved AP2/ERF DNA-binding domain, critical for recognizing cold-responsive gene promoters, further validates the functional homology of this gene across taxa. Phylogenetic clustering with *Brassica napus* and *B. oleracea* suggests that *P. hysterophorus* exploits ancient genetic pathways to colonize thermally variable environments, a strategy that may explain its global proliferation.

Structural modeling of the DREB1/CBF protein revealed a stable globular structure with solvent-exposed β-sheets, a feature consistent with its role in DNA binding. The refined model's high validation scores (ERRAT: 95.6%, Ramachandran favored regions: 93.3%) confirm its reliability for future studies, such as molecular docking experiments to identify potential inhibitors for weed control.

While this study advances our understanding of cold adaptation in invasive species, certain limitations must be acknowledged. The short cold-exposure period may not fully replicate natural frost conditions, and focusing solely on *DREB1/CBF* overlooks potential interactions with other stress-responsive genes. Future work should explore transcriptomic and proteomic networks to unravel broader regulatory mechanisms.

Table 1. BLASTn hits for *P. hysterophorus DREB1/CBF* exhibit homology with associated species. The table shows high sequence similarity with *DREB1/CBF* gene family members in Brassica species. Remarkably, some entries have similar percent identity values such as *Brassica napus* (two different entries; BNCBF5 and BnDREB1B, but same species) are because of high *DREB1/CBF* genes high conservation across species, so, they exhibit the same identity score to *PhCBF/DREB1*.

Species' genes	Species scientific names	E-Value	Per. Identity	Accession numbers
PhCBF/DREB1	*Parthenium hysterophorus*	-	-	-
BrDREB1B	*Brassica rapa*	4e-34	90.25%	KJ013589.1
BnCBF5	*Brassica napus*	3e-141	91.25%	AF499031.1
BoDREB1B	*Brassica oleracea*	9e-141	91.25%	XM013770359.1
BnDREB1B	*Brassica napus*	9e-141	91.25%	XM013828057.3

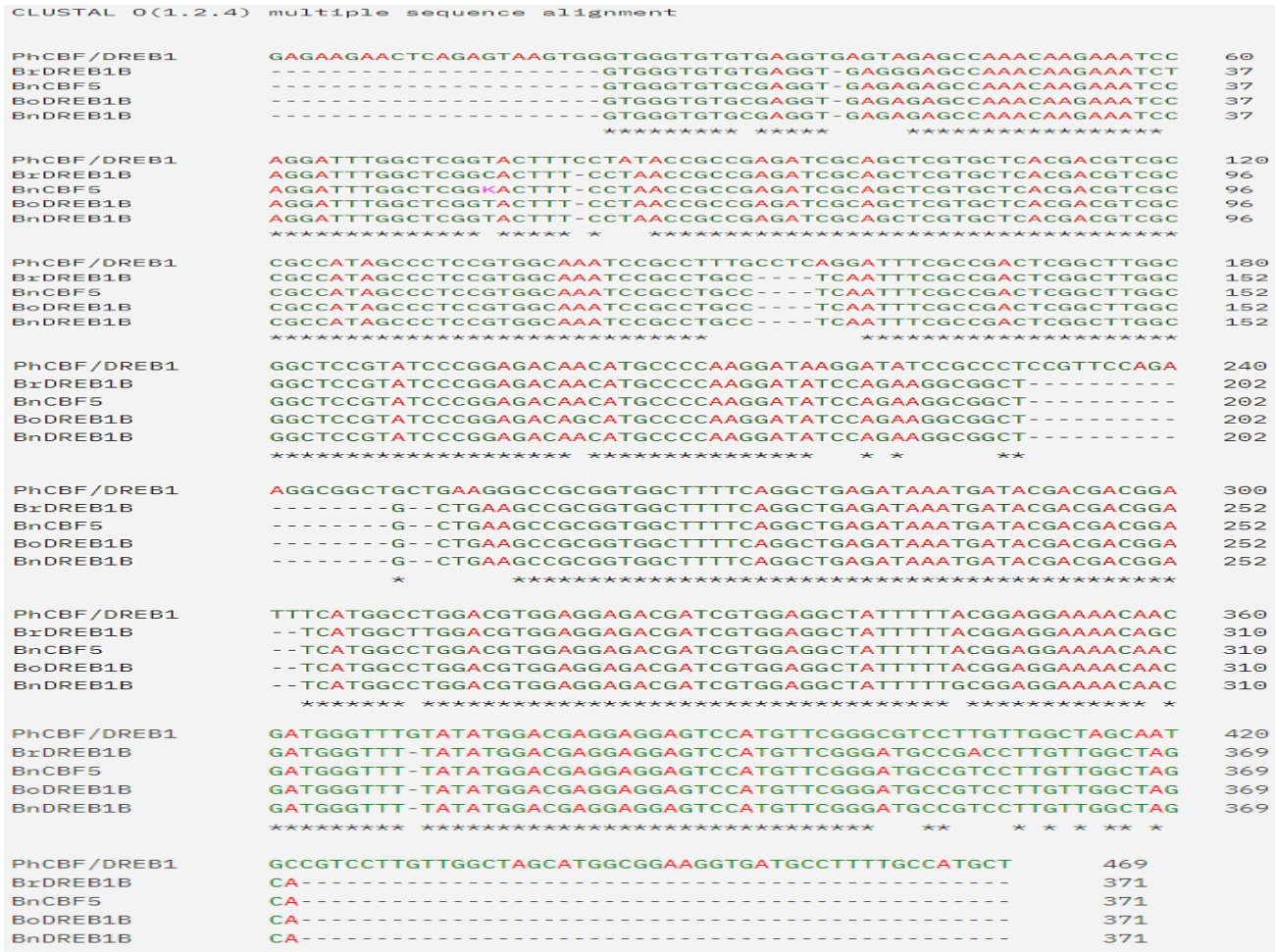

Fig. 4A. Multiple sequence alignment of *P. hysterophorus DREB1/CBF* (AN335566) with homologous sequences from *Brassica napus* (AF499031), *Brassica oleracea* (XM013770359), and *Brassica rapa* (KJ013589) using ClustalW2. Conserved AP2/ERF domains are highlighted.

Fig.4B. Amino acid sequence alignment (BLASTx) indicating conserved residues (red) and amino acid substitutions (blue), reflecting evolutionary divergence.

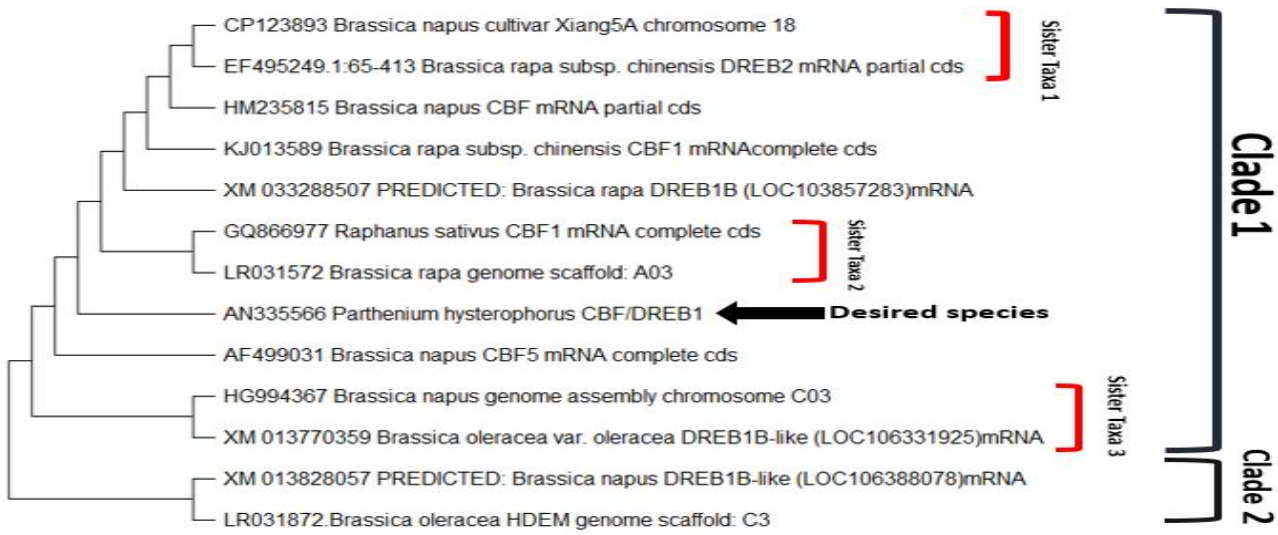

Fig. 4C. Phylogenetic Relationships of *DREB1/CBF* Homologs. A phylogenetic tree constructed using the Neighbor-Joining method (MEGA11) based on 494 nucleotide positions. Bootstrap values (>70%) are displayed at branch points. *P. hysterophorus* (black arrow) clusters closely with *Brassica* species, indicating evolutionary conservation. Scale bar represents nucleotide substitutions per site. Accession numbers: *B. napus* (AF499031, XM013828057), *Raphanus sativus* (GQ866977), *B. oleracea* (XM013770359).

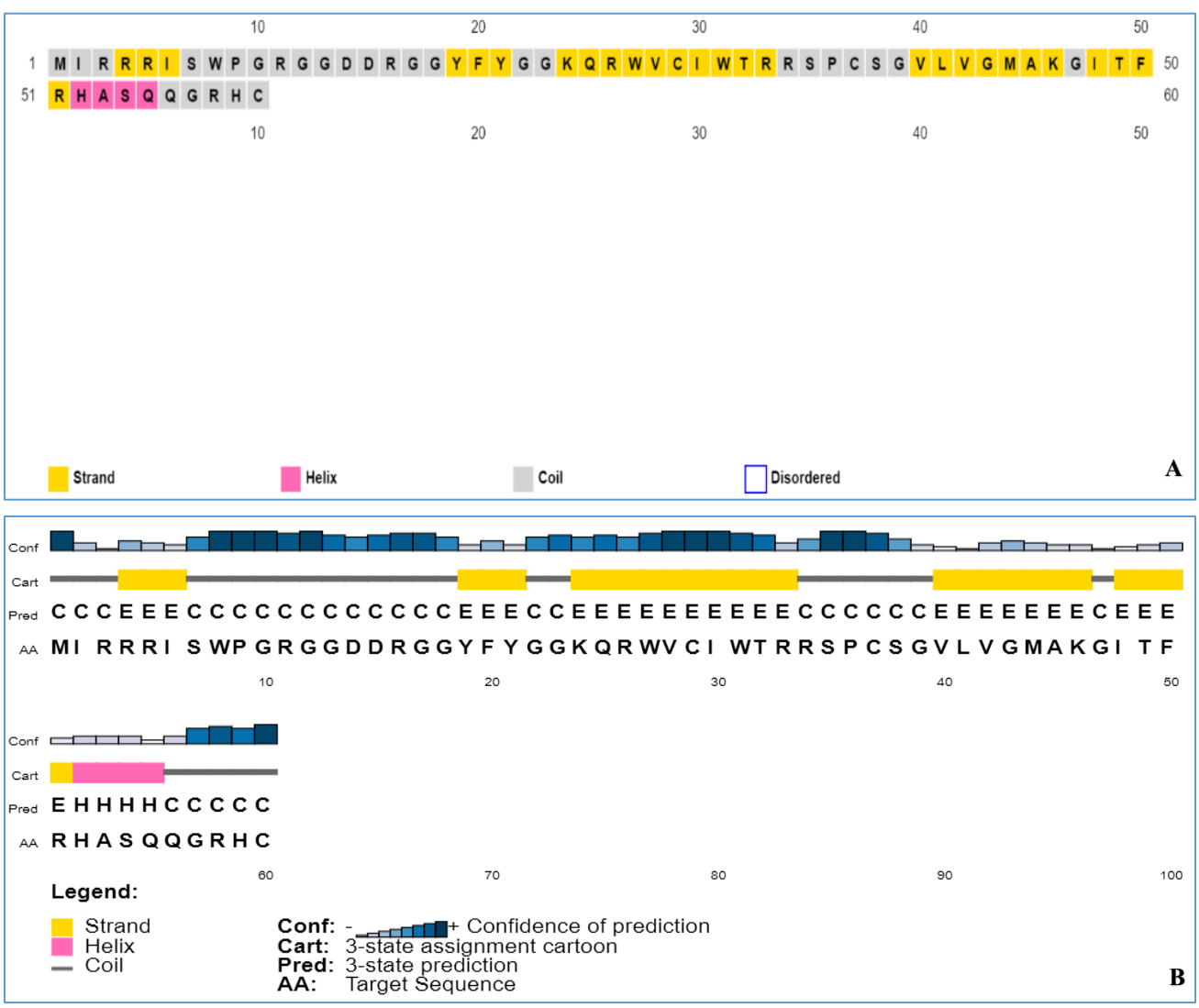

Fig. 5(A-B). Predicted Secondary Structure of *DREB1/CBF* Protein. (A) PSIPRED-generated secondary structure prediction displaying α-helices (pink), β-strands (yellow), and coils (gray). Identified structural elements include β-strands at residues 4–6 (β1), 19–21 (β2), 24–33 (β3), 40–46 (β4), 48–51 (β5), and an α-helix at residues 52–55 (α1). (B) Structural representation highlighting PSIPRED confidence scores, where darker shades indicate greater prediction reliability.

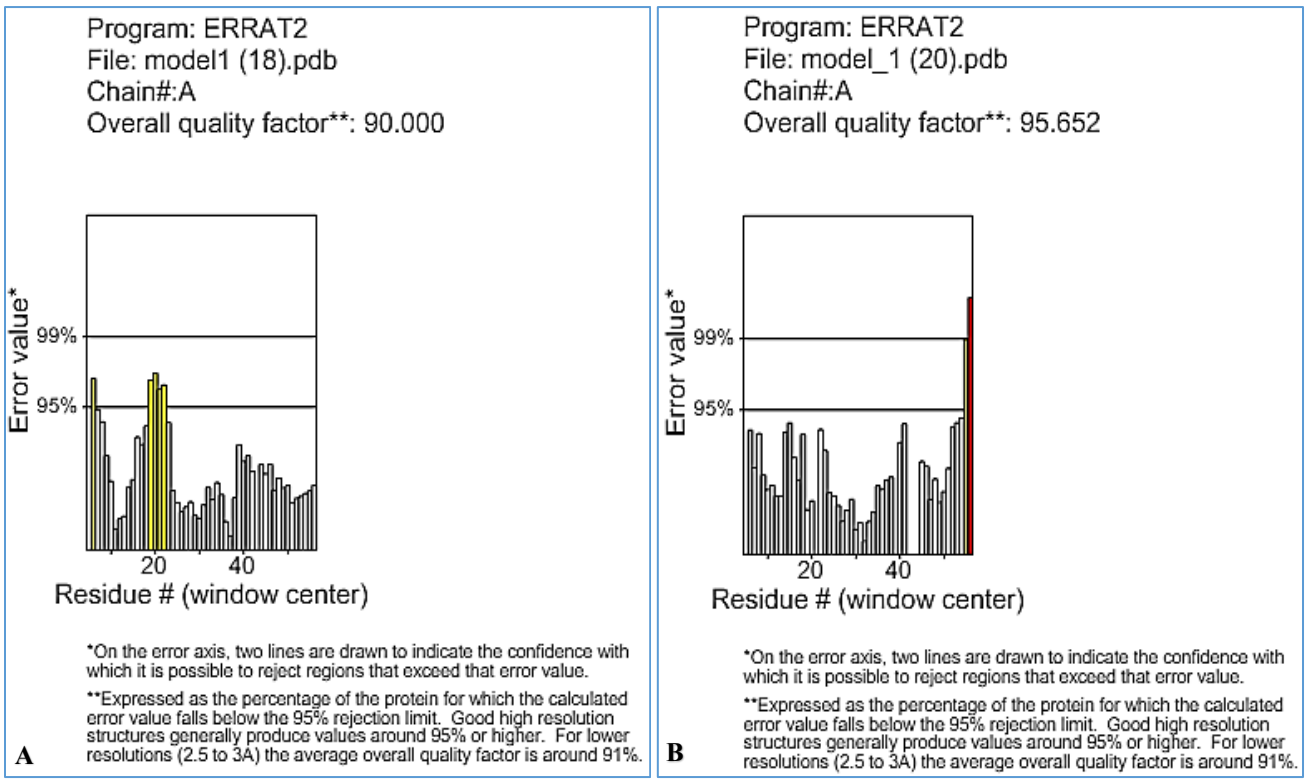

Fig. 6(A-B). Structural Validation of *DREB1/CBF* 3D Models Using ERRAT. (A) Initial protein structure model generated by trRosetta, with an ERRAT overall quality score of 90. (B) Post-refinement model using GalaxyRefine, improving the quality score to 95.6. The dashed line represents the 85% acceptability threshold, demonstrating structural optimization through refinement.

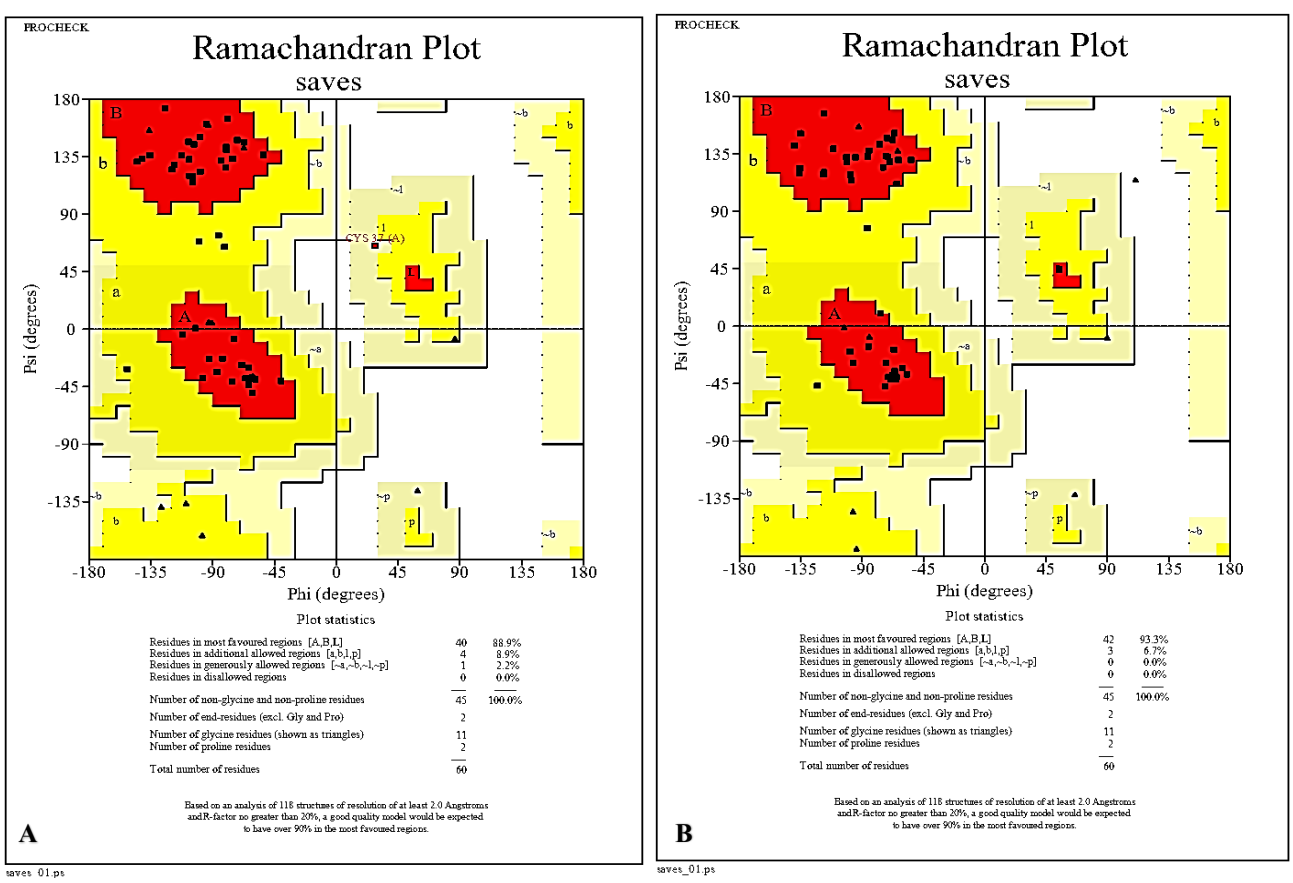

Fig. 7(A-B). Ramachandran Plot Assessment of Structural Stability. (A) Ramachandran plot of the initial model, indicating 88.9% residues in favored regions (dark blue), 8.9% in allowed regions (light blue), and 2.2% in generously allowed regions (pale blue). (B) Refined model exhibits improved stability with 93.3% residues in favored and 6.7% in allowed regions. Backbone dihedral angles (φ, ψ) were analyzed using PROCHECK.

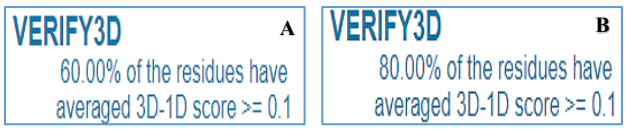

Fig. 8(A-B). Verify3D Model Compatibility Analysis. (A) Initial 3D model assessment showing that 60% of residues scored above the acceptable threshold (>0.2), indicating structural inconsistencies. (B) After refinement, the model demonstrated 80% compatibility, confirming improved structural integrity. Verify3D scores were determined by comparing the 3D model's atomic environment to its amino acid sequence.

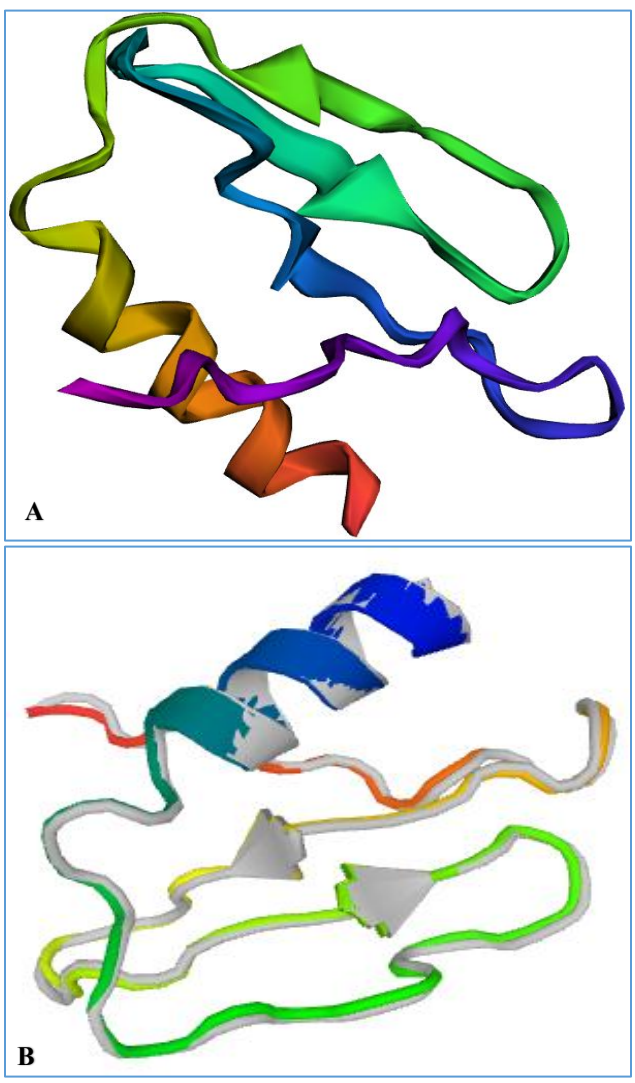

Fig. 9. Predicted 3D structure of protein (A) before refinement and (B) Post-Refinement.

Conclusions and Future recommendation

This study reveals that *Parthenium hysterophorus* combats frost stress by activating the *DREB1/CBF* gene, which strengthens cell membranes (reducing damage by 56%) and elevates protective carotenoid levels (20% increase) to counteract cold-induced harm. The gene shares striking genetic similarities (>90%) with cold-tolerant *Brassica* species, suggesting plants across families use shared mechanisms to survive freezing temperatures. Its protein structure, confirmed through advanced modeling, is stable and functional, enabling it to regulate genes critical for cold adaptation. These insights propose two pathways for practical application: first, disrupting *DREB1/CBF* in *P. hysterophorus* through gene-silencing techniques could reduce its invasive spread, and second, introducing this gene into crops may bolster their frost resistance, protecting food supplies in colder climates. Future efforts should prioritize field trials to test these strategies, assess ecological risks of genetic modifications, and explore how *DREB1/CBF* interacts with other genes to refine stress-response models. This dual approach addresses both ecological conservation and agricultural resilience in an era of climate uncertainty.

Declaration: The authors declare no conflicts of interest.

Author's Contributions: Adnan Jamil: Methodology, Formal analysis, Investigation, Writing Original Draft, Fazal Hadi: Conceptualization, Resources, Writing-Review and Editing, Funding acquisition, Supervision, Jamil Ahmad: Softwares, Formal analysis**,** Nasir Ali: Writing-Review and Editing.

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