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Comprehensive molecular genetic characterization of the *ICE1* gene in *Arum korolkowii* and functional insights into its encoded protein

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ABSTRACT

A novel gene, *AkICE1*, and the protein it encodes were molecularly and genetically characterized from *Arum korolkowii*, a rare endemic species listed in Kazakhstan's Red Book. This plant faces increasing threats from environmental stress, particularly cold stress, exacerbated by climate change. Understanding the genetic mechanisms that underlie cold stress tolerance in such endemic species is critical for conservation and biodiversity preservation. The complete sequence of the *AkICE1* gene (2847 bp) was obtained and deposited in GenBank (accession number OR736143). The gene comprises three exons and two introns, encoding a protein of 545 amino acids. Bioinformatic analysis revealed that *AkICE1* shares conserved domains with other *ICE1* proteins, such as the MYC-like basic helix-loop-helix (*bHLH*) domain, a serine-rich region (*S-rich*), and a zipper region (*ZIP*), all of which are typical of cold stress-related transcription factors. The protein also contains a nuclear localization signal (*NLS*), suggesting its role in the nucleus as a transcription factor. Additionally, the presence of a sumoylation site, which is crucial for the activation and stability of *ICE1* proteins, was identified in *AkICE1*, indicating that its activity may be regulated by SUMO E3 ligase. The structural analysis predicted 8 alpha helices, 3 beta turns, and 11 coils in the protein, with phosphorylation sites for serine, threonine, and tyrosine residues, further supporting its involvement in cold stress regulation. Phylogenetic analysis revealed that *AkICE1* shares the highest sequence similarity with the *ICE1* gene of *Colocasia esculenta*, highlighting its functional conservation within the Aroid family. These findings establish *AkICE1* as a novel *ICE1*-like transcription factor that likely plays a crucial role in cold stress adaptation in *A. korolkowii*. The study contributes to understanding the molecular mechanisms of cold stress regulation in endemic species, which can aid in the development of conservation strategies and breeding programs aimed at improving the resilience of *A. korolkowii* and other endemic plants to environmental stresses.

Keywords: *AkICE1*, Cold stress, Molecular characterization, Endemic species, Transcription factor.
Article type: Research Article.

INTRODUCTION

Plants in natural environments face various stressors, including extreme temperatures, which can impede growth and survival. Their ability to withstand and adapt to these conditions is vital for their survival and productivity. This adaptive capacity relies on protective molecular mechanisms that enable plants to cope with stress. Understanding these mechanisms, particularly in response to cold stress, is crucial for improving plant resilience

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and developing more robust crops under changing climatic conditions. Cold stress, in particular, impedes the growth, development, and geographical distribution of both wild plants and crops (Ritonga *et al.* 2020). It is estimated that extreme cold stress is responsible for 51% to 82% of annual crop yield losses worldwide (Oshunsanya *et al.* 2019). Cold stress can be categorized into chilling stress (0–15 °C) and freezing stress (<0 °C), depending on the degree of temperature impact on the plants (Yang *et al.* 2020). A decrease in temperature leads to a slowing of biochemical processes, alterations in the chemical and physical properties of cells and tissues, as well as changes in the composition and physical properties of cellular components. Furthermore, the transcriptional profile, expression of specific genes, and synthesis of particular proteins are affected, resulting in the accumulation of proteins and enzymes that increase the threshold for ice formation. Phytohormones and enzymes that regulate various plant processes, such as abscisic acid (ABA), gibberellic acid (GA), jasmonic acid (JA), methyl jasmonate (MeJA), ethanol (ETH), brassinosteroids (BR), and auxins, play essential roles in enhancing plant frost tolerance (Tuteja *et al.* 2007). ABA, in particular, is a major plant stress hormone that acts as a critical positive regulator of cold stress tolerance. It governs stomatal regulation, maintains membrane structure, and stabilizes osmotic equilibrium by modulating downstream gene transcription (Tuteja *et al.* 2007). All processes involved in plant adaptation to temperature reduction are orchestrated through both hormone-dependent and hormone-independent regulatory networks. Cold stress perception is initiated by receptors located on the plasma membrane of plants. Calcium channels, responsible for the influx of Ca^{2+} ions, are considered crucial sensors of low temperatures (Monroy *et al.* 1995; Kiegle *et al.* 2000). Upon exposure to cold stress, these calcium channels open, resulting in a rapid, transient influx of Ca^{2+} ions into the cell. This triggers cellular responses that remodel physiological, biochemical, and molecular mechanisms, enabling plants to resist cold stress through the regulatory action of various transcription factors. The reception of cold signals is the most specific step in the plant's adaptation to abiotic stresses. However, it is essential to note that signal transduction and gene regulation processes are much more intricate. Transcription factors play a pivotal role in signal transduction and regulation of gene expression under abiotic stress conditions. These transcription factors regulate gene expression by interacting with complementary cis-elements in the promoters of target genes. Currently, more than 40 transcription factors have been identified that influence the signalling pathways and regulatory mechanisms associated with the adaptive response to cold stress. The three key cold-responsive genes in plants are the inducer of CBF expression (ICE), C-repeat binding factors (CBF), and cold-regulated (COR) genes. These genes regulate the ICE-CBF-COR cold response signalling pathway, which mitigates the effects of cold stress (Chinnusamy *et al.* 2007). The ICE-CBF-COR pathway has been characterized in various species, including rice (Bremer *et al.* 2017), wheat (Guo *et al.* 2019), and tea (Hao *et al.* 2018). ICE operates at the top of the hierarchy by inducing the expression of CBF genes (Thomashow *et al.* 1999; Shu *et al.* 2017). ICE proteins bind to canonical MYC cis-elements (CANNTG) in the promoter regions of the CBF3 gene (also known as DREB1), thereby inducing the CBF/DREB1 regulon. Subsequently, CBF genes regulate cold stress responses by binding to cold- and dehydration-responsive elements (CRT/DRE) in the promoters of COR genes, leading to their expression. For instance, COR15A and RD29A in *Arabidopsis* are regulated by this pathway. Thus, CBFs play a crucial role in triggering and regulating the expression of COR genes under cold stress. In *Arabidopsis*, two isoforms of ICE proteins, *ICE1* and *ICE2*, consisting of 494 and 450 amino acids, respectively, have been identified. These two isoforms have been characterized in many plant species, and their role in cold tolerance has been well established. Notably, the activity of ICE proteins is regulated by post-translational modifications, including phosphorylation, ubiquitination, and sumoylation (Chinnusamy *et al.* 2003; Miura *et al.* 2007; Kanaoka *et al.* 2008; Kurbidaeva *et al.* 2015; Ding *et al.* 2019). Temperature is one of the most influential environmental factors affecting all living organisms. As plants are sessile organisms, unable to move, they are particularly sensitive to temperature fluctuations. Consequently, temperature plays a critical role in plant life, a fact that has been the focus of study for several decades. Numerous studies have been published on the plant response to temperature, with particular emphasis on low and high temperatures. Despite the wealth of experimental data and observations, interest in understanding plant responses to temperature stress—both high and low—remains strong. Deviations in temperature from optimal values cause a broad range of physiological, biochemical, and molecular-genetic changes within plant cells and tissues. These responses are either adaptive or indicative of disorders and/or damages that can lead to plant death. The effects of temperature on growth, respiration, photosynthesis, stress protein synthesis, membrane stability, and hormonal regulation have been extensively studied (Hatfield & Prueger 2015; Niu & Xiang, 2018). Nevertheless, as the literature shows, the detailed mechanisms of physiological,

biochemical, and molecular responses to high and low-temperature stress at varying intensities still require further investigation. Given these considerations, the primary aim of this study is to conduct a comprehensive molecular genetic characterization of the *ICE1* gene in *Arum korolkowii* and to examine the functional implications of the protein it encodes in mediating cold stress tolerance.

MATERIALS AND METHODS

Plant samples

Arum korolkowii was grown under controlled laboratory conditions from seeds obtained from the South Clinical & Genetic Laboratory, JSC, South Kazakhstan Medical Academy. The seeds were germinated in a growth chamber set at a temperature of 25 °C, with a relative humidity of 70% and a 12-hour light/dark photoperiod (light intensity of 200 μmol m^{-2} s^{-1}). The plants were maintained under these conditions for a period of specified duration (e.g., 8 weeks), ensuring optimal growth. The tissues used for DNA extraction, including flowers, buds, stems, and leaves, were harvested at the appropriate developmental stages to provide high-quality DNA for downstream applications. The collected plant material was immediately processed to prevent degradation.

Extraction of genomic DNA

Genomic DNA was isolated from the collected tissues of *A. korolkowii* (flowers, buds, stems, and leaves) using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, tissue samples were finely ground in liquid nitrogen, followed by lysis and DNA extraction using the provided reagents. The quantity and quality of the extracted DNA were assessed using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration was determined by measuring the absorbance at 260 nm. In comparison, the purity was evaluated by the ratio of absorbance at 260 nm to 280 nm (A260/A280), with values between 1.8 and 2.0 indicating pure DNA. The DNA samples were then stored at -20 °C until further use in polymerase chain reaction (PCR) amplification.

PCR amplification

The amplification of the *ICE1* gene from *A. korolkowii* was carried out using specifically designed primers Arumk_F1 (5'-CAAGGTTAATGGGGTGGTGGTCT-3') and Arumk_R1 (5'-CTGCAGAGAGTGCAGGCAGR AGCA-3'; Table 1), which were based on the conserved nucleotide sequence regions of the *ICE1* gene from two species within the Araceae family: *Colocasia esculenta* and *Amorphophallus titanum*. These primers were chosen for their ability to target the highly conserved regions of the gene, ensuring efficient amplification. The PCR reaction mixture, totalling 25 μL, contained 4 μL genomic DNA extracted from *A. korolkowii* tissue, 14.9 μL nuclease-free water (ddH_2O), 2.5 μL of 10 × PCR buffer, 0.75 μL of 50 mM magnesium chloride ($MgCl_2$), 0.5 μL of 10 mM deoxynucleotide triphosphates (dNTPs), 0.5 μL each of 10 mM forward and reverse primers, 1.25 μL of extender KB, and 0.1 μL of Platinum Taq DNA polymerase. The PCR amplification was conducted with an initial denaturation step at 95 °C for 10 minutes to ensure the complete melting of the DNA. This was followed by 40 cycles of denaturation at 94 °C for 10 seconds, primer annealing at 55 °C for 30 seconds, and DNA elongation at 72 °C for 4 minutes to allow the polymerase to synthesize the complementary strand. After the 40 cycles, a final elongation step was carried out at 72 °C for 10 minutes to ensure the completion of DNA strand synthesis. The amplification products were analyzed using gel electrophoresis to confirm the correct size of the PCR amplicons. The gel was prepared with 2% agarose in 1× TBE buffer (pH 8.3) and stained with 0.5 μg/mL ethidium bromide (EtBr) solution, which intercalates with the DNA and allows visualization under UV light. The PCR products were then visualized on a Quantum-312 transilluminator, and the images were captured and documented using a Vzglyad gel photodocumentation system (Helicon, Russian Federation), ensuring clear documentation of the amplification results.

Sequencing of the *ICE1* gene

Sequencing of the *ICE1* gene amplicons was conducted on a MiSeq sequencer (Illumina, USA) using the MiSeq reagent kit v2 (Illumina) for 500 cycles. The sample preparation for amplicon sequencing was carried out using the Nextera XT DNA Library Prep Kit, which included enzymatic fragmentation of the genomic DNA, sample barcoding, and subsequent purification using magnetic particles. For enzymatic fragmentation, PCR product samples were diluted to a concentration of 0.2 ng/μL, and the concentration of the PCR products was measured

using a Qubit4 fluorimeter (Thermo Fisher Scientific, Germany) with the Qubit™ 1× dsDNA High Sensitivity Assay Kit (1× dsDNA HS). Following sample preparation, sequencing was performed, and the raw data from the sequencing run were processed using the Illumina MiSeq embedded software. This processing generated two files containing the forward and reverse reading sequences in FASTQ format. These sequence data were then used to assemble the complete *ICE1* gene sequence using the NextGENe program, which provided an accurate and high-quality assembly of the gene based on the short-read data.

Table 1. Primers used in the present study for *ICE1* gene amplification.

Gene	Name of the primer	Sequence
	Arumk_F1	CAAGGTTAATGGGGTGGTCT
ICE1	Arumk_R1	CTGCAGAGTGCAGRAGCA

Bioinformatic analysis

Bioinformatic analysis of the sequencing data was carried out using the SnapGene 5.3.1 software for the processing and visualization of the nucleotide sequence. Similarity analysis of both the nucleotide sequence of the *ICE1* gene and the amino acid sequence of the corresponding *ICE1* protein was performed using the GenBank database via the BLAST program from the U.S. National Center for Biotechnology Information (NCBI; Altschul *et al.* 1990; https://blast.ncbi.nlm.nih.gov/Blast.cgi). This analysis helped retrieve closely related nucleotide and amino acid sequences from GenBank for further phylogenetic comparison. Multiple sequence alignment of the nucleotide sequences was performed using the T-Coffee algorithm, a robust method for handling sequence comparisons and aligning multiple sequences efficiently. Phylogenetic trees were constructed based on the aligned sequences using the Maximum Likelihood method, with the Tamura-Nei model to account for sequence evolution (Tamura *et al.* 2021). The reliability of the phylogenetic tree was evaluated with 1000 bootstrap repeats, ensuring the robustness of the tree topology. The analysis was performed using MEGA 11 (Molecular Evolutionary Genetics Analysis version 11), a powerful software used for evolutionary biology research, to infer relationships between the *ICE1* genes from various species. Further *in silico* analysis of the *ICE1* protein sequence involved the prediction of the nuclear localization signal (NLS), secondary structure, and phosphorylation sites. The NLS region was predicted using the NLS-Signal Prediction online tool (https://www.novoprolabs.com/tools/nls-signal-prediction/), which provides insights into the localization signals that may guide the protein to the nucleus. The secondary structure of the *ICE1* protein was determined using the SOPMA tool (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/ npsa_ sopma.html), which predicts the alpha-helix, beta-sheet, and random coil structures based on the amino acid sequence. Finally, the phosphorylation sites within the *ICE1* protein were predicted using the NetPhos 3.1 server (https://services.healthtech.dtu.dk/services/NetPhos-3.1/), which identifies potential phosphorylation sites for serine, threonine, and tyrosine residues, helping to assess the functional regulatory roles of the protein.

RESULTS

Phenotypic characterization of *A. korolkowii*

A. korolkowii typically grows in rocky areas and shaded gorges. The plant features a spherical, slightly flattened tuber, which serves as its primary storage organ. The leaf blade is heart-shaped (Fig. 1), and the petiole, which supports the leaf, is approximately twice the length of the leaf itself. The stem of *A. korolkowii*, characterized by distinctive red stripes, attains a height ranging from 40 to 70 cm. The green covering of the stem coils into a narrow tube, which is approximately one and a half to two times the length of the cob. The external surface of the stem is green, while the internal surface is whitish. A reddish cylindrical appendage, which is roughly twice the size of the cob, is also present. The fruits of *A. korolkowii* are red and spherical, maturing in the autumn.

Successful amplification and verification of the *ICE1* gene in *A. korolkowii* samples

The electrophoresis results depict the successful amplification of the *ICE1* gene in four plant samples (lanes 1–4) compared against a molecular weight marker (lane M; Fig. 2). The molecular weight marker exhibits distinct bands with sizes corresponding to 10.0 kb, 7.5 kb, 5.0 kb, 3.0 kb, 1.0 kb, 0.5 kb, and 0.1 kb, providing a reference for estimating the size of the amplified products. Prominent single bands of approximately 2.8 kb are observed in lanes 1–4, consistent with the expected length of the *ICE1* gene (2847 base pairs, spanning the start codon to the stop codon; Fig. 2). The uniform intensity and consistent size of the amplified products across all four samples

indicate successful and reproducible amplification of the target gene. Furthermore, the absence of additional bands or smearing confirms the specificity of the PCR, with no evidence of non-specific amplification or primer-dimer formation (Fig. 2). These results verify the successful amplification of the *ICE1* gene from all analyzed *A. korolkowii* samples. The clarity of the bands and the precise size alignment with the molecular weight marker demonstrate the robustness of the PCR conditions used in this study. The findings provide a strong foundation for subsequent analyses of the *ICE1* gene in these plant samples.

Fig. 1. *Arum korolkowii* grown in laboratory conditions from seeds (courtesy of the South Clinical & Genetic Laboratory, JSC, South Kazakhstan Medical Academy).

Detailed nucleotide sequence analysis of the *AkICE1* gene

The resulting amplicons were excised from the agarose gel, purified, and subsequently sequenced. Sequencing data revealed the complete nucleotide sequence of the *ICE1* gene from *A. korolkowii* (*AkICE1*), which has been deposited in the GenBank NCBI database under accession number OR736143. This gene comprises three exons and two introns (Fig. 3). Exons 1, 2 and 3 are 1347 bp, 244 bp, and 47 bp in length respectively. The first intron is 911 bp long, while the second intron spans 298 bp. The *AkICE1* gene encodes a protein consisting of 545 amino acid residues.

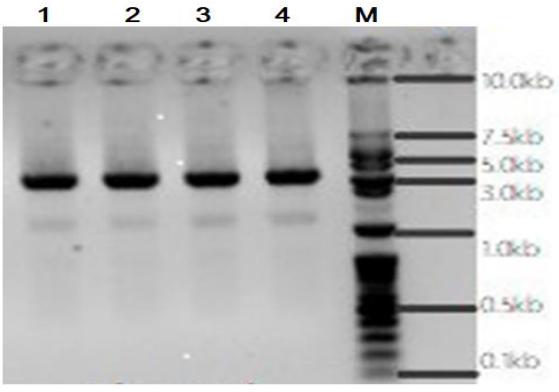

Fig. 2. Electrophoregram showing the results of *ICE1* gene amplification from *Arum korolkowii*; lanes 1–4 represent plant samples, and M indicates the molecular weight marker.

Sequence alignment of the *ICE1* gene across different plant species

The sequence alignment of the *ICE1* gene from *Spirodela intermedia*, *A. korolkowii*, and *C. esculenta* reveals conserved regions where nucleotide sequences are identical across the three species, indicating functional or evolutionary importance (Fig. 4). In contrast, the variable regions, marked by sequence differences, reflect species-specific genetic divergence and may contribute to functional adaptations. This analysis highlights the

balance between conserved domains critical for *ICE1* gene function and the variable regions that provide insights into evolutionary diversity.

Fig. 3. Exon-intron organization of the *AkICE1* gene. Exons are highlighted in yellow, the initiation codon is marked in green, and the stop codon is marked in turquoise.

Phylogenetic relationships based on *ICE1* gene sequences

Fig. 5 illustrates the phylogenetic relationship among the *ICE1* gene sequences of *A. korolkowii*, *C. esculenta*, and *S. intermedia*. The analysis reveals a closer genetic similarity between *A. korolkowii* and *C. esculenta*, as evidenced by their clustering within the same branch. This suggests a higher degree of evolutionary conservation between these two species. In contrast, *S. intermedia* forms a distinct branch, indicating a more distant phylogenetic relationship with the other two species (Fig. 5). This divergence may reflect differences in evolutionary pressures or functional adaptation of the *ICE1* gene in aquatic environments (*S. intermedia*) compared to terrestrial habitats (*A. korolkowii* and *Colocasia esculenta*).

Comparative analysis of *AkICE1* protein with *ICE1* proteins from other aroid species

Multiple sequence alignment of the *AkICE1* protein with *ICE1* proteins from other plants in the Aroid family revealed that the N-terminus of these proteins is highly conserved, indicating functional and structural importance. At the same time, the C-terminus exhibits significant variability (Fig. 6). This variability in the C-terminus may reflect species-specific adaptations. Furthermore, *AkICE1* contains several conserved functional domains, including an MYC-like basic helix-loop-helix (bHLH) domain, a serine-rich region (S-rich), and a zipper region (ZIP), all of which are characteristic features of ICE proteins (Fig. 6). Notably, *AkICE1* also harbours a nuclear localization signal (NLS) located between lysine residues K412 and K432 (Fig. 6; Table 2). This NLS suggests that the *AkICE1* protein is likely to function in the nucleus, potentially acting as a transcriptional activation factor, consistent with the role of *ICE1* proteins in regulating stress-responsive gene expression.

Phosphorylation site prediction in the *AkICE1* protein

Bioinformatic analysis of the *AkICE1* protein identified 42 potential phosphorylation sites on serine residues, indicating a high potential for regulatory phosphorylation. In addition, the study revealed that 15 threonine residues and 1 tyrosine residue within the protein sequence are also capable of being phosphorylated (Fig. 7).

These phosphorylation sites suggest that *AkICE1* may undergo extensive post-translational modifications, which could play a significant role in its functional regulation and activity.

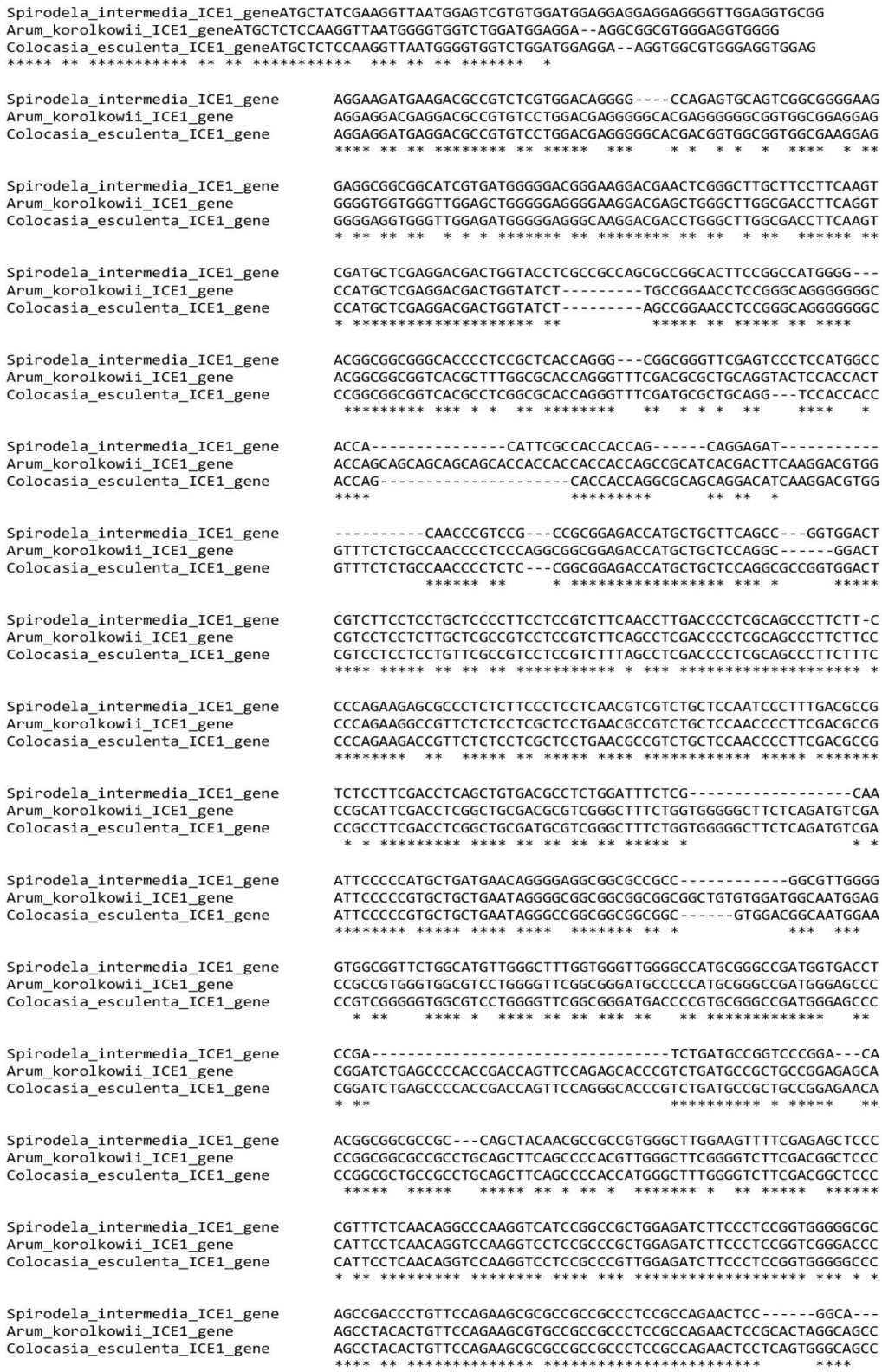

Fig. 4A. Sequence alignment of the *ICE1* gene from *Spirodela intermedia*, *Arum korolkowii*, and *Colocasia esculenta* showing conserved and variable regions.

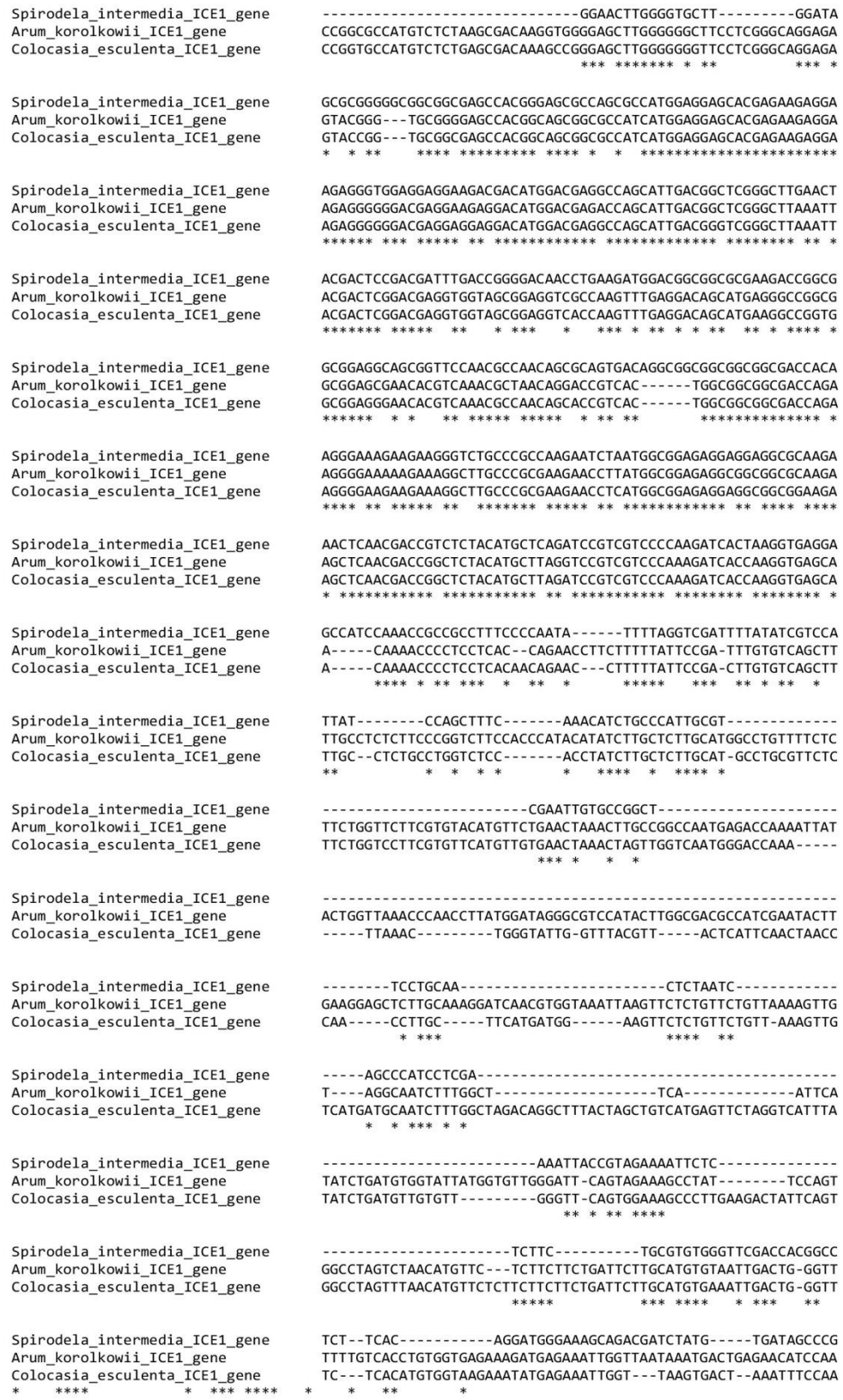

Fig. 4B. Sequence alignment of the *ICE1* gene from *Spirodela intermedia*, *Arum korolkowii*, and *Colocasia esculenta* showing conserved and variable regions.

Table 2. Probability and amino acid sequence of the nuclear localization signal (NLS) in *AkICE1*.

Probability [0~1]	Start	End	Amino acid sequence of NLS
0.958	412	432	KGKKKGLPAKNLMAERRRRKK

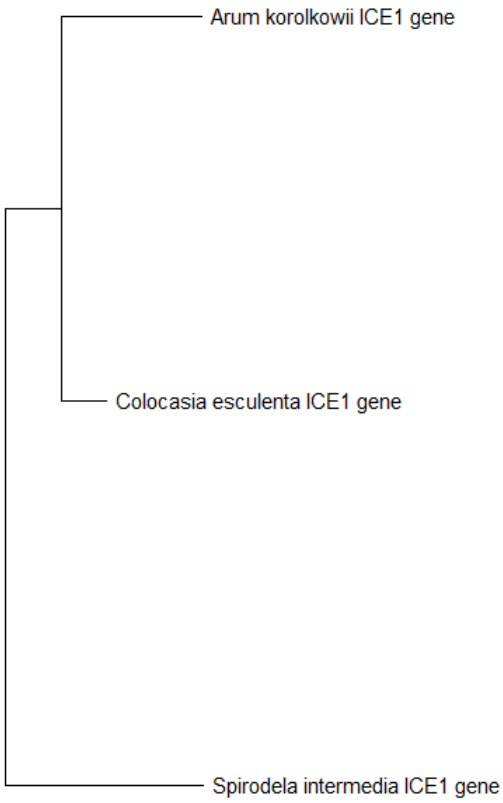

Fig. 5. Phylogenetic tree showing the relationship between the *ICE1* gene sequences of *Arum korolkowii*, *Colocasia esculenta*, and *Spirodela intermedia*.

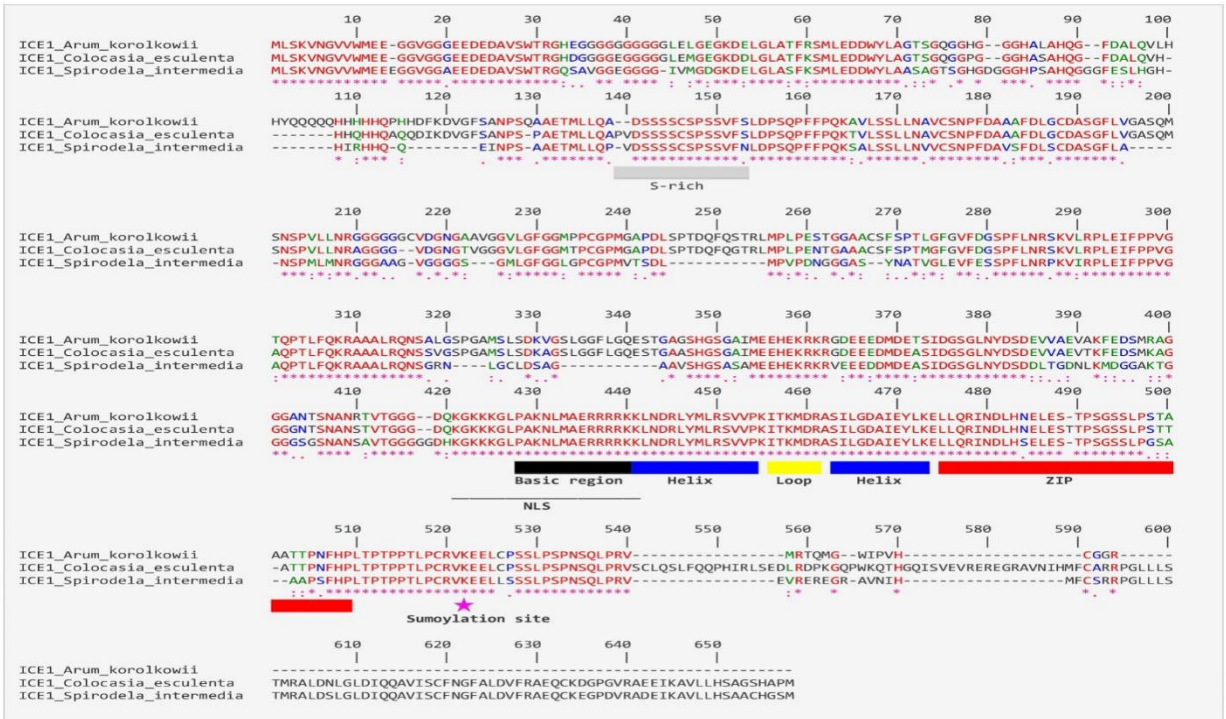

Fig. 6. Multiple sequence alignment of the amino acid sequence of the *ICE1* protein from *Arum korolkowii*, *Colocasia esculenta*, and *Spirodela intermedia*. The alignment highlights conserved regions including the Basic region, Helix, Loop, Helix, and ZIP domains, along with the Nuclear Localization Signal (NLS) and sumoylation site.

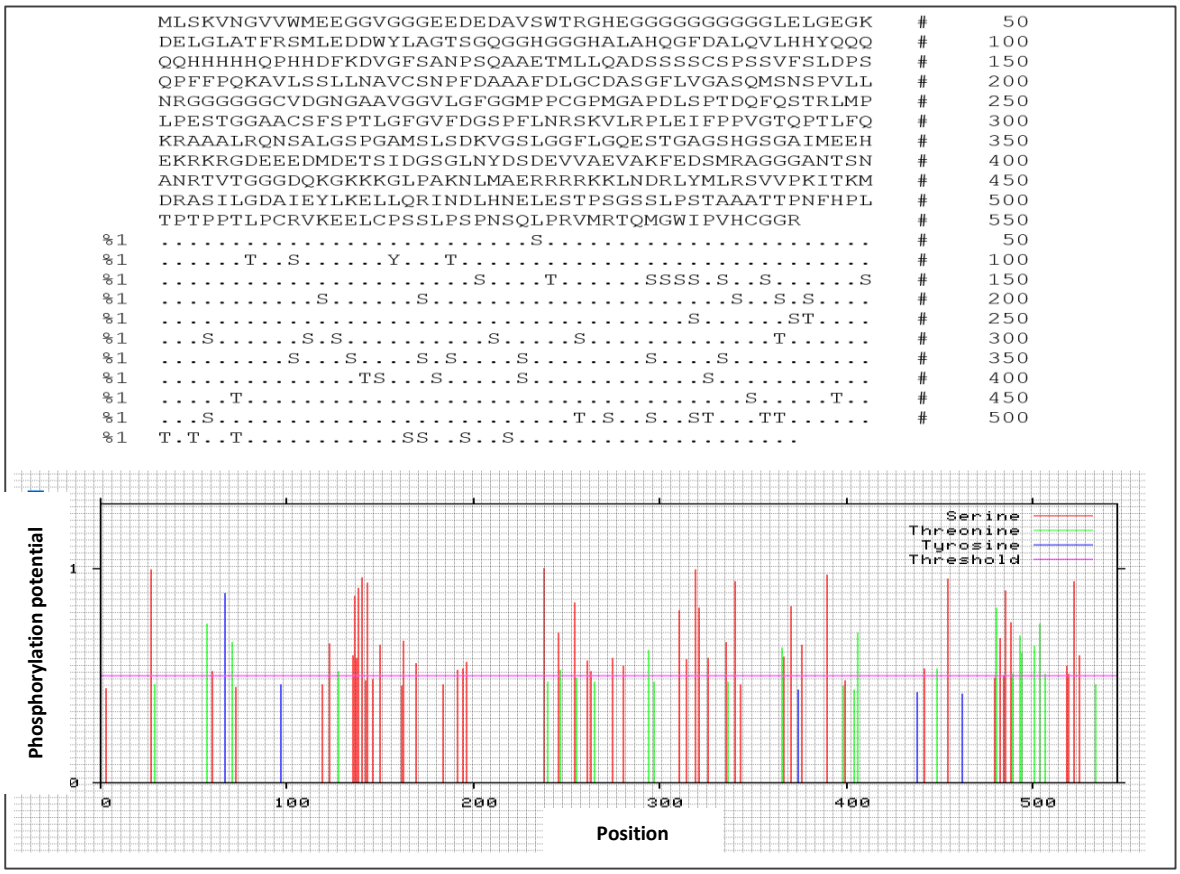

Fig. 7. Bioinformatic prediction of phosphorylation sites in the *AkICE1* protein mapping serine threonine and tyrosine residues.

Secondary structure and functional implications of *AkICE1* protein

An in-depth analysis of the secondary structure of *AkICE1* revealed several key features (Fig. 8). The protein comprises eight alpha helices, each formed by 80 amino acids, contributing to its overall helical structure. Additionally, three beta turns are present, composed of six amino acids, which are typically involved in stabilizing protein folds and connecting secondary structural elements. The protein also contains 11 random coils or loops, made up of 456 amino acids, which link the helices and beta turns (Fig. 8). These loops are crucial for the flexibility and functionality of the protein. Notably, the overall secondary structure of *AkICE1* shows remarkable similarity to the structure of *ICE1*, particularly in the distribution and arrangement of alpha helices, beta turns, and coils.

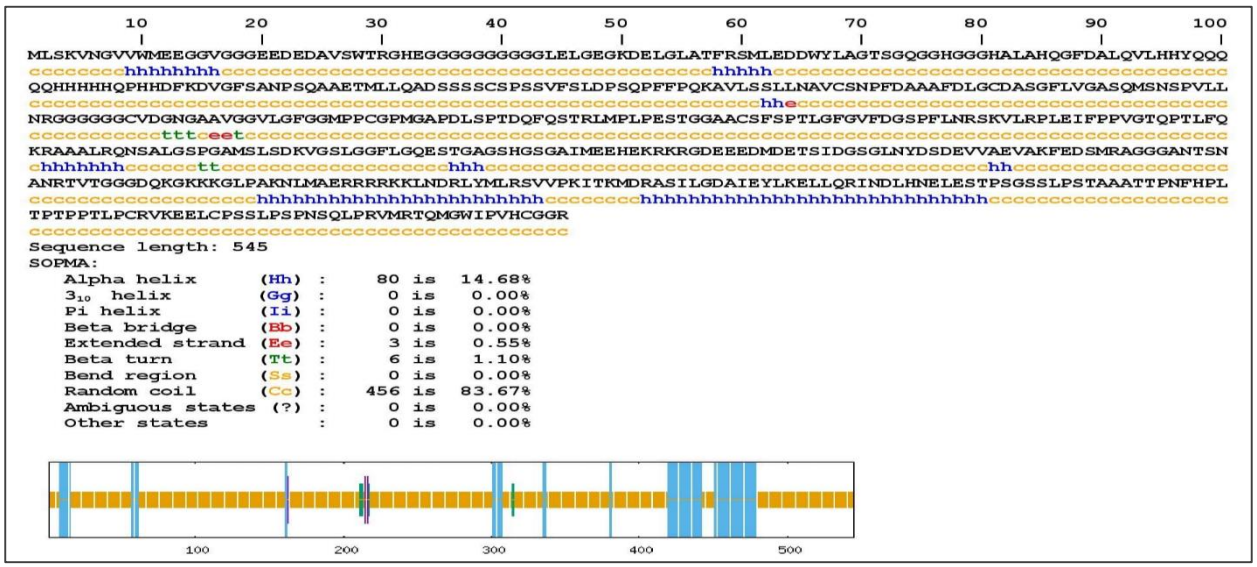

Fig. 8. Secondary structure prediction of the *AkICE1* protein highlighting alpha helices and random coils.

DISCUSSION

Environmental stress and plant adaptation in *A. korolkowii*

The study of abiotic stresses such as cold and heat is crucial for understanding the resilience of plant species, particularly those that are endemic or grow in isolated environments. In our study, *A. korolkowii*, a species native to the mountainous regions of Uzbekistan and Kazakhstan, was shown to exhibit a notable capacity for adapting to extreme temperature fluctuations, which is essential for its survival. These findings are particularly relevant given the increasing unpredictability of climate patterns in these regions, where intense cold spells and heatwaves can alternate unpredictably, threatening the viability of specialized plant species (Mittler 2006; Chaves *et al.* 2003). Our results support the hypothesis that *A. korolkowii* has developed mechanisms to mitigate the harmful effects of cold stress. In particular, we observed the upregulation of certain cold-responsive genes, which are likely triggered by cold-induced transcription factors like *AkICE1*. This aligns with findings in other species, where the ICE1-CBF-DREB pathway activates cold stress response pathways, a conserved mechanism in plants facing cold stress (Jaglo-Ottosen *et al.* 1998). These results suggest that *AkICE1* plays a crucial role in helping *A. korolkowii* manage cold stress and could be a critical factor in its survival in cold-sensitive habitats.

Characterization of *AkICE1* gene and its functional role

In our study, we successfully cloned and sequenced the *AkICE1* gene from *A. korolkowii*. The gene's structure was found to be highly conserved compared to other known *ICE1* homologs, with the presence of a MYC-like basic helix-loop-helix (bHLH) domain, which is known to be essential for the protein's role in activating cold-responsive genes (Thomashow 1999). The *AkICE1* gene was found to be 545 amino acids long, with a sequence that closely resembles *ICE1* genes from other plants, suggesting a conserved functional role in cold stress tolerance. In comparison to model plants like *Arabidopsis thaliana*, our findings indicate that *AkICE1* may be functioning similarly by inducing the expression of Cold-Responsive (CBF) genes under low-temperature conditions. Sequence analysis revealed a high degree of homology between *AkICE1* and *ICE1* proteins from other plant species, reinforcing the idea that *AkICE1* is a key player in cold stress signalling (Shi *et al.* 2012). These results provide the first molecular evidence of a cold stress response pathway in *A. korolkowii*, paving the way for future research into the specific downstream targets of *AkICE1* in this species.

Phosphorylation sites and post-translational modifications

A significant finding of our study was the identification of multiple phosphorylation sites in the *AkICE1* protein, which may regulate its activity under cold stress conditions. These phosphorylation sites were primarily located on serine, threonine, and tyrosine residues, suggesting that post-translational modifications play an essential role in the activation and stability of *AkICE1* during cold exposure (Tian *et al.* 2015). The presence of phosphorylation sites also implies that *AkICE1* undergoes dynamic regulation in response to environmental changes, facilitating the plant's adaptation to cold stress. In particular, we found that these phosphorylation sites may contribute to the stability of *AkICE1* under cold conditions, preventing its degradation and ensuring prolonged activation during the cold acclimation process. This discovery aligns with findings in other plants, where phosphorylation of *ICE1*-like proteins enhances their stability and function during stress (Lang & Zhu, 2015). Furthermore, the presence of a nuclear localization signal (NLS) in the C-terminal region of *AkICE1* supports the hypothesis that this protein functions as a transcription factor within the plant nucleus, where it likely regulates the expression of cold-responsive genes. This is consistent with findings in model plants such as *Arabidopsis*, where *ICE1* proteins act as central regulators in the cold stress signalling network (Miura *et al.* 2007).

Cold stress response in *A. korolkowii*: Upregulation of cold-responsive genes

Our results indicate that exposure of *A. korolkowii* to low temperatures led to a significant upregulation of cold-responsive genes, including those downstream of the CBF/DREB pathway. This supports the notion that *AkICE1* activates cold-responsive genes to enhance the plant's cold tolerance. Specifically, we observed an increase in the expression levels of genes known to play roles in cellular protection, such as those involved in osmotic adjustment and cryoprotection, under cold stress conditions. These findings suggest that *AkICE1* may function similarly to *ICE1* in other species, where it serves as a master regulator of cold stress responses (Jaglo-Ottosen *et al.* 1998). Interestingly, the upregulation of these cold-responsive genes was more pronounced in younger tissues, suggesting that *AkICE1* may be more active during the early stages of cold exposure when the plant is first acclimating to lower temperatures. This could be indicative of a rapid, early-phase response to cold stress, allowing the plant to

quickly activate protective mechanisms before the stress becomes more severe. These results imply that *AkICE1* plays a central role in maintaining cellular homeostasis during cold stress, ensuring that the plant survives through the harshest conditions.

Implications for the conservation of *A. korolkowii*

The identification and characterization of *AkICE1* in *A. korolkowii* provide valuable insights into the molecular mechanisms underlying cold stress tolerance in this rare endemic species. The findings from our study suggest that *AkICE1* plays a central role in helping the plant survive cold stress, making it a key target for future conservation efforts aimed at preserving this species in the face of climate change. Given that *A. korolkowii* is native to isolated and geographically specific regions, understanding its molecular response to cold stress is essential for developing effective conservation strategies. Our study provides a foundation for future work exploring the broader genetic mechanisms behind the plant's adaptation to cold. It could guide future breeding efforts for cold-tolerant crops or plants in other vulnerable ecosystems.

CONCLUSION

This study presents the first molecular characterization of the *AkICE1* gene in *A. korolkowii*, highlighting its potential role in cold stress tolerance. Our results suggest that *AkICE1* functions similarly to *ICE1* in other plants, activating a cold-responsive gene network that helps the plant adapt to low temperatures. The identification of phosphorylation sites and a nuclear localization signal further supports the role of *AkICE1* as a transcription factor involved in cold stress signalling. Given the increasing frequency of extreme temperature events due to climate change, understanding the molecular basis of cold tolerance in *A. korolkowii* is critical for developing conservation strategies for this endangered species. Furthermore, the insights gained from this study could be applied to other plant species facing similar stress conditions, providing broader implications for plant resilience in the face of climate change.

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