

Molecular genetic analysis of the ICE1 gene in *Arum korolkowii* Regel

Aigerim Yeginbay^{1,2*}, Gulzhaina Alpamysova¹, Zhanar Yelemanova¹, Zauze Narymbayeva¹, Amina Daulbay¹, Rakhat Pernebekova², B. A. Abduvaliev³, Altunbek Burabaev⁴, Assilbek Burabaev⁴, Abdujalil Narimanov³

1. Department of Biotechnology, Higher School of Chemical Engineering and Biotechnology, M. Auezov South-Kazakhstan University, Tauke-Khan, Shymkent, 160000, Kazakhstan

2. International Kazakh-Turkish University named after H.A.Yasavi, Turkestan city

3. Institute of Genetics and Plants Experimental Biology, Academy of Sciences of Uzbekistan, Tashkent Region, Kibray District, Yuqori-Yuz, 111226 Uzbekistan

4. South Clinical and Genetic Laboratory, South Kazakhstan Medical Academy, Shymkent, 160000, Kazakhstan

* Corresponding author's Email: aigerimgray@gmail.com

ABSTRACT

The paper presents a molecular genetic analysis of the expression of the ICE 1 gene *Arum korolkowii* Regel involved in the abiotic stress response in the Korolkov Aronnik in cold and frost conditions. The classical PCR method was used to identify the ICE 1 gene in Aronnik Korolkov and sequenced this site to determine the uniqueness of this gene. When stress is induced, increased expression of the studied gene is shown. It has been proved that the expression of this gene in Aronnik Korolkov begins already at the stage of acclimatization.

Keywords: ICE1 gene, *Arum korolkowii* Regel, Plants, Kazakhstan, PCR, Sequence.

Article type: Research Article.

INTRODUCTION

The beginning of the 20th century was characterized by a powerful breakthrough in the development of scientific and technological progress, the growth of social contradictions, a sharp demographic explosion and deterioration of the human environment. Truly, our planet has never been subjected to such physical overloads before. Human has never before levied so much tribute from nature and has never been so vulnerable to the power that he himself created. As a result, at the beginning of the twentieth century, people faced a lot of environmental and nature management problems. Desertification, urbanization, exploration and extraction of minerals, as well as pollution of ecosystems. This is not a complete list of the problems of humanity of the 21st century. The central place in this list is occupied by the problem of the disappearance of rare plant species (Vinogradov 2009; Yeginbay *et al.* 2022, 2023). For a long time man has been closely interacting with nature. However, this relationship does not always have a good effect on the flora and fauna of nature. A large number of plants were destroyed by people themselves. In addition, at the end, we realized that if something is not done, then our children and grandchildren will not find many different types of flora. The most effective measure was the creation of an annotated list of rare animals and plants, which was called the Red Book, since red is a symbol of trouble, danger and offensive aggression (Vorontsova 2005; Vinogradov 2009; Yeginbay *et al.* 2022, 2023). The Red Book is a collection of facts about the unique inhabitants of our planet, over which there is a serious threat of extinction. The book itself does not protect; it only tells about those species that are on the verge of extinction. The sacred duty of all citizens of the World is to protect the riches of the earth. Among the most important riches is its vegetation cover. In addition, although many plants are still not used by humans, it should be emphasized that the disappearance of each biological species from the face of the Earth is very often an irreparable loss, both for science and practice. In total, about 350 thousand plant species live on the planet, striking people with a variety of their forms. At least one plant species disappears from the face of the Earth irrevocably every year. In modern practice, a significant

number of plants are still used, among which there are very ancient and very rare species. They have been preserved thanks to human. To date, many valuable plants have disappeared as a result of human activity. This is evidenced by the results of preliminary counts of extinct species conducted in different countries. So, in the UK, the list of rare and endangered plants includes 300 species, in New Zealand it has 314 species. Currently, about 20 thousand plant species are under threat of extinction on the Globe (Altschul *et al.* 1990; Vorontsova 2005; Vinogradov 2009; Yeginbay *et al.* 2022). In the last 50 years, there has been a mark of this species. This is due to many factors, among which the main contribution is made by a person, followed by cattle grazing, flower picking due to decorative and medicinal properties and other human activities, In addition to a decrease in the level occurring due to several amounts of pollinating antibiotics and external weather conditions, such as the duration and frosty winters (Yeginbay *et al.* 2022, 2023). Molecular genetic studies of these species have a special meaning for solving the main problems in detention conditions. Major discoveries in biology (identification of DNA structure, polymerase chain reaction, creation of DNA chips, sequencing), as well as significantly increased technical capabilities allowed the formation of the science of molecular biology and a whole group of methods known as molecular or molecular genetic methods. Currently, these methods are actively used not only in biology, but also in other fields including medicine, criminology, archaeology. Modern biology cannot be imagined without molecular genetic methods. The first were methods based on protein polymorphism. Isoenzyme analysis makes it possible to identify the level of polymorphism of populations, their genetic structure, to determine the level of genetic closeness or, conversely, openness of populations, and much more. Currently, these methods continue to be used, however, most researchers and laboratories are switching to the analysis of nucleic acid polymorphism. This is partly due to the fact that proteins can be isolated only from living material or from frozen at a temperature of -70 to -80 °C, whereas DNA can be isolated from both living and dried material. In addition, DNA analysis methods make it possible to study both nuclear and plastid and mitochondrial genomes. So, molecular genetic methods based on polymerase chain reaction and DNA sequencing are actively used in such areas of plant research as: (i) genetic polymorphism of natural populations; (ii) analysis of purity of varieties; (iii) identification of hybrids; (iv) identification of phylogenetic relationships of species, genera and taxa of a higher level; and (v) analysis of transcriptional activity genes. Despite the harsh climatic conditions, the flora of Kazakhstan is striking in its diversity. But more and more plants are becoming unique, including because of their small number. You need to know the Red Book representatives in order not to commit irreparable through ignorance. The International Union for Conservation of Nature has determined the gradual death of the animal and plant world. In this regard, the idea arose to study each plant individually from the list of the Red Book of Kazakhstan, which are under threat. You need to know the Red Book representatives in order not to commit irreparable through ignorance. In addition, the first of the plants we had the opportunity to study Aronika Korolkova from the list of Red Book plants. Resolution of the Government of the Republic of Kazakhstan dated October 31, 2006 No. 1034 approved a new list of plants listed in the Red Book of the country. The list includes 373 species of plants, 13 species of fungi and one species of lichens. Among them Korolkov's aronnik can be found among the rocks and shady gorges of Central Asia, China and Iran. Based on the above, it can be said that the study of any kind of Red Book plants is relevant not only in the territory of the Republic of Kazakhstan, but also throughout the world. In this connection, we had the opportunity to study among the Red Book medicinal plants the Aronnik of Korolkov living on the territories of the Republic of Kazakhstan.

MATERIALS AND METHODS

The object of the study was 3-month artificially grown (on the basis of seeds) Red Book medicinal plants of Aronnik Korolkov (from the collection of the South clinical & Genetic Laboratory of JSC at South Kazakhstan medical academy): Aronnik Korolkov in the amount of 50 pcs. and a wild species that grows in forest areas in the amount of 50 pcs (Jaborova *et al.* 2023). Laboratory plants were grown in a volume of 2-L brown forest soil (pH 5.0). Prior to the induction of low-temperature exposure, the plants were kept in laboratory conditions for a month at a temperature of 20 ± 2 °C with an optimal watering regime and illumination with fluorescent lamps, a photoperiod of 16/8 with an illumination intensity of 3000 lux. Afterward, leaves were taken from them for analysis. Then these plants were placed in cold chambers. Stress induction was carried out by exposure to temperatures up to $0 \dots +2$ °C for seven days (cold stress), followed by a decrease in temperature from -4 to -6 °C for five days (freezing), the light regime was kept the same. For laboratory analyses at all stages of the study,

the third (for RNA isolation) and fourth (for physiological analyses) leaves from above were used. To isolate the RNA, each of the three repetitions was a mixed sample of leaves from the Korolkov Aronnik.

Isolation of genomic RNA from biomaterials by Aronnik Korolkov. The biological material for RNA isolation were flower, bud, stem, leaf and root in the size of 1-2 m² with minimized damage to the Korolkov Aronnik. To analyze gene expression, RNA was isolated using a commercial kit (Qiagen, USA) according to the manufacturer's instructions. The integrity of the isolated RNA was tested by electrophoresis in 1% agarose gel. The amount of RNA was determined spectrophotometrically on a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Synthesis of the first cDNA chain

The first cDNA chain was synthesized using Promega reagents and protocol in a volume of 20 µL. The resulting cDNA was diluted to the final cDNA concentration in a solution of 12.5 ng µL⁻¹.

Analysis of relative gene expression

PCR was performed on Mastercycler egradient S and Mastercycler ProS amplifiers by Eppendorf with a set of reagents for conducting basic reactions in a volume of 25 µL. The sequences of primers were taken from literature sources, so that only cDNA would react. The list of primers is given in Table 1. The synthesis of primers was carried out on the DNA/RNA Synthesizer H8 "Germany".

The following program was used for amplification: 1 cycle of 10 min at 95 °C; 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The specificity of amplification was verified by analyzing the melting curve and visualizing products in agarose gel.

Table 1. Primers used in the experiment.

| Gene | Name of the primers | Sequence | Source |
|------|---------------------|------------------------------|-------------------|
| ICE1 | ICE1-1f | 5'-CCCATTAAAACAGCTGATCACA-3' | Kurbidaeva (2015) |
| | ICE1-r | 5'-CCAGCAAGCTAGAGTTGAGGTT-3' | |

Electrophoresis in agarose gel

Electrophoresis in 1.5% agarose gel in the presence of ethidium bromide (0.1 mg mL⁻¹) was used to separate the amplification products. An electrophoretic chamber was used for horizontal electrophoresis. It was performed in a TBE buffer (100 mM Tris/ HCl pH 8.0; 10 mM EDTA; 2% SDS). DNA sequencing was performed in the CCP—Genome. Polymorphic sites were visually checked on chromatograms and confirmed by comparing forward and reverse reads. Sequences with E-value ≤ 1e⁻⁰⁵ were used, and redundant sequences were deleted. The list of sequences is given in Table 2.

Table 2. Primers for sequencing the ICE 1 gene.

| Gene | Name of the primer | Appointment | Sequence | Source |
|------|--------------------|-------------|------------------------|----------|
| ICE1 | Pr_ICE1_F1 | Sequencing | TGTGAGAGATGCTVTCSAAGGT | Primer 3 |
| | Pr_ICE1_F2 | | ATGGCGGAGAGGAGGCG | Primer 3 |
| | Pr_ICE1_R3 | | TTGACCGCYCGGCCTTC | Primer 3 |
| | Pr_ICE1_R1 | | CTACATSGRRSRTGGC | Primer 3 |
| | Pr_ICE1_R2 | | GYTSTCACCTTGGTGATCTT | Primer 3 |

Methods of bioinformatics of Ortholo proteins 1 and 2 in the Phytosome v9.1 database (<http://www.phytozome.net/>), GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and UniProt (<http://www.uniprot.org/>) using the BLASTP algorithm (Altschul *et al.* 1990); <http://blast.ncbi.nlm.nih.gov>. The nucleotide material ICE1 versatile ras was searched in the database 1001 gene (<http://1001genomes.org/>). Visualization and comparison of results in the BioEdit v7 program 2.0 (Hall 1999), numerous comparisons of results using ClustalW (Thompson *et al.* 1994), polymorphism analysis - DnaSP 5.2 (Rozas *et al.* 2023), DNA Slider (McDonald 1998) and UGENE v1.11.5 (Okonechnikov *et al.* 2012). For genetic analysis, the methods of maximum training and user integration were used (Saitou & Nei 1987) in the MEGA 5.2 package (Saitou & Nei 1987; <http://www.megasoftware.net>), using a p-distance correlation model and a Poisson substitution model (Tian *et al.* 2023). The secondary structure and conserved domains of the protein were studied using PSIPRED (Tamura *et al.* 2011), (<http://bioinf.cs.ucl.ac.uk/psipred/>), Emboss sepestfind (Flint & Kendler 2014; <http://emboss.bioinformatics.nl/cgi-bin/emboss/epstfind>).

NLStradamus (<http://www.moseslab.csb.utoronto.ca/NLStradamus/>), Poly(A) Signal Miner (Nomura *et al.* 2023; <http://dnafminer.bic.nus.edu.sg/PolyA.html>), KinasePhos2.0 (Wong *et al.* 2007; <http://kinasephos2.mbc.nctu.edu.tw/index.html>), MyHits (Pagni *et al.* 2007; <http://myhits.isb-sib.ch/>), KEGG Genes Database (Kanehisa *et al.* 2002; <http://www.genome.jp/kegg/genes.html>), UniProtKB (Magrane 2011; Narimonov *et al.* 2023; <http://www.uniprot.org/uniprot/>). Bandit was used to register the nucleotide sequences of genes in GenBank NCBI (<http://www.ncbi.nlm.nih.gov/BankIt/>). Statistical data processing was carried out using Microsoft Office Excel and STATISTICA 6.1 (StatSoft. Inc.) programs (Jaborova *et al.* 2020).

RESULTS

As a result of RNA isolation, the following frequency was obtained, which was determined spectrophotometrically on a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA; Tables 3 and 4).

Table 3. Quantitative indicator of isolated RNA.

| Nº of samples | ng μL^{-1} | A260/A280 |
|---------------|-----------------------|-----------|
| 1 | 8.5 | 1.22 |
| 2 | 4.1 | 1.25 |
| 3 | 7.2 | 1.33 |
| 4 | 9.0 | 0.96 |
| 5 | 11.4 | 0.90 |
| 6 | 7.9 | 1.02 |
| 7 | 3.1 | 9.72 |
| 8 | 4.3 | 1.29 |
| 9 | 4.6 | 1.59 |
| 10 | 2.1 | 7.56 |
| 11 | 0.4 | -1.06 |

Table 4. Quantitative indicator of isolated RNA for sequencing

| Sample | ng μL^{-1} | A260/A280 |
|--------|-----------------------|-----------|
| ICE1 | 6.7 | 1.58 |
| | 9.7 | 1.51 |

The results of the PCR study showed that the ICE1 cold resistance genes are present in the studied plants of the Korolkov Aronnik. Although the presence of these genes fluctuates a lot with expressiveness on cold stress. When comparing the cold/frost response, significant differences were observed in the expression of the ICE 1 genes (Fig. 1).

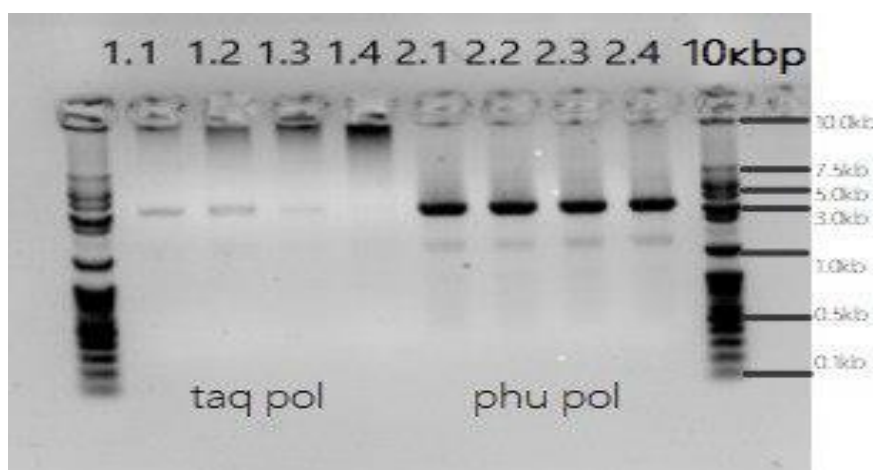


Fig. 1. 10 kbp – Molecular weight marker (1.1-1.4 cold and 2.1-2.4 frost) ICE1 gene.

From the results obtained, it can be seen that frost exposure enhances the expression of the ICE 1 gene in *Arum korolkowii*.

Structural features of the ICE 1 gene

The ICE2 gene is localized on the 3rd chromosome in reverse orientation, this gene consists of 4 exons and 3 introns (Fig. 2). ICE1 proteins consist of 494 amino acid residues.

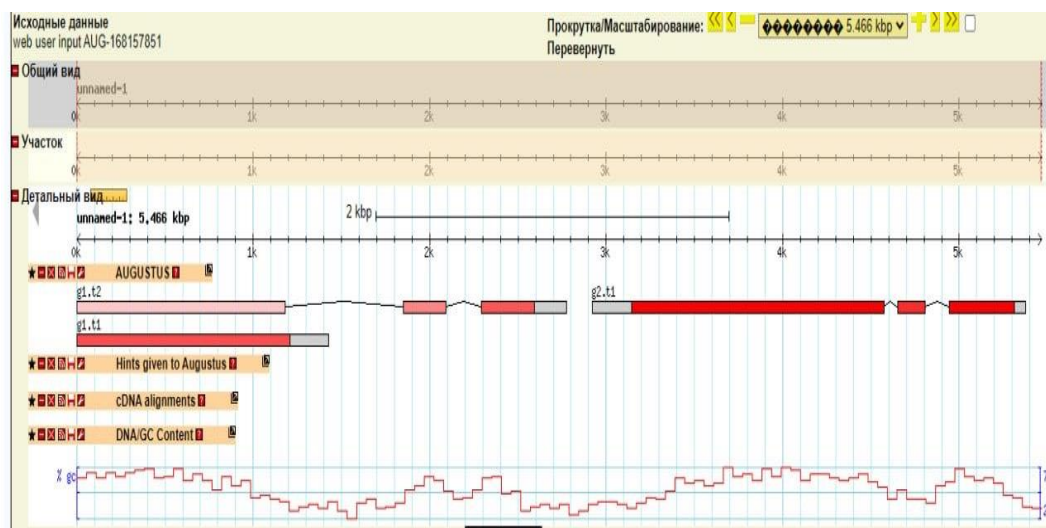


Fig. 2. Structural characteristics of the ICE 1 gene.

Using computer modeling of the secondary structure of the protein, it was found that the domains form additional alpha helices. Thus, the structural divergence of ICE1 sequences is revealed.

Using the sequencing methods of the ICE 1 gene, a huge sequence of the site > *Arum korolkowii* ICE1 gene 2999 bp was obtained.

The sequenced section of the ICE 1 gene was sent for publication to the international genbank (<http://www.ncbi.nlm.nih.gov>) which is under consideration. LOCUS Seq18 2999 bp DNA linear PLN 24-OCT-2023

DEFINITION ICE1 gene, complete cds.

ACCESSION Seq18

VERSION

KEYWORDS

SOURCE: *Arum korolkowii*

ORGANISM: *Arum korolkowii*

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliopsida; Liliopsida; Araceae; Aroideae; Areae; Arum.

REFERENCE 1 (bases 1 to 2999)

AUTHORS: Yeginbay, A, Adilov, B, Sherimbetov, AG, Burabaev, A, Ruzmetov, DR, Abduvaliev, B, Burabaev, A and Narimanov, AA.

TITLE: Direct submission

JOURNAL: unpublished

REFERENCE 2 (bases 1 to 2999)

AUTHORS: Yeginbay, A, Adilov, B, Sherimbetov, AG, Burabaev, A, Ruzmetov, DR, Abduvaliev, B, Burabaev, A & Narimanov, AA.

TITLE: Direct Submission

JOURNAL: Submitted (24-OCT-2023) Laboratory of plant immunity and Department of Biotechnology, Institute of Genetics and PEB, M. Auezov South Kazakhstan University, Yukori-yuz, Kibray district, Tashkent region 111226, Uzbekistan, Kazakhstan

COMMENT Bankit Comment: ALT EMAIL: sheranvar@mail.ru

Bankit Comment: TOTAL # OF SEQS: 1

##Assembly-Data-START##

Sequencing Technology: Sanger dideoxy sequencing

##Assembly-Data-END##

FEATURES: Location/Qualifiers

source 1..2999

```

/organism = "Arum korolkowii"
/mol_type = "genomic DNA"
/db_xref = "taxon:578871"
/country = "Kazakhstan"
/collection_date = "2022"
gene      1..2803
/gene = "ICE1"
CDS      join(1..1614,2259..2498,2678..2803)
/gene = "ICE1"
/codon_start = 1
/product = "ICE1"

```

The phylogenetic tree was constructed using the maximum likelihood method (ML) and neighbor joining (NJ). Topology of dendrograms was based on sequences of ICE-1 proteins (Fig. 3).

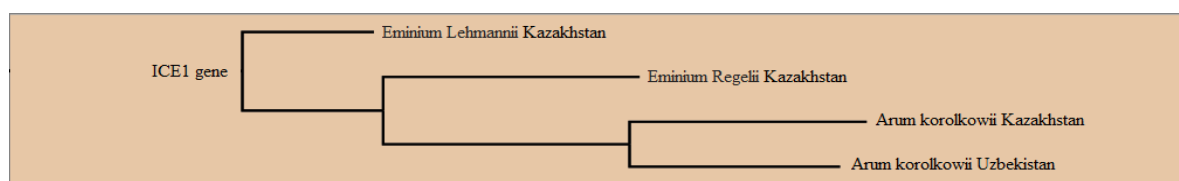


Fig. 3. ICE-1 dendrogram of similar plant proteins. The tree was built using the NJ algorithm in the MEGA 5 program 2.

Based on the results obtained, first of all, there is a clear separation of the *Arum korolkowii* family, forming a separate cluster. In general, as shown in Fig. 1, the studied plants form three groups of clusters according to the ICE gene: one of the branches is formed by two *Arum* (*Arum korolkowii* Kazakhstan and *Arum korolkowii* Uzbekistan), which is explained by the genetic proximity of these families.

The second branch is *Eminium regelii* Kazakhstan.

The third is formed by *Eminium lehmannii* Kazakhstan.

Here, additional research is needed to clarify some historical facts about the origin of these plants.

CONCLUSION

Many of the species listed in the Red Book of Kazakhstan meet all the criteria of the international requirement, which increases their vulnerability and conservation value. This work is dedicated to Veronika Karalkoova and is aimed at maximally studying their genetic structure and uniqueness with the use of modern equipment. The study of *Arum korolkowii* by one ICE-1 gene can already be considered a victory and the beginning of the study. However, scientists face a huge way to study all the genes of this unique plant.

The preservation of Red Book plants is necessary for the future of the younger generation.

1. According to phylogenetic analysis, based on the ICE 1 gene, *Arum korolkowii* Kazakhstan and *Arum korolkowii* Uzbekistan are the closest families.
2. A characteristic of the structural features of the ICE 1 gene is given.
3. Based on the obtained PCR results, the relative fluctuations in the expression of the ICE 1 gene *Arum korolkowii* Kazakhstan on cold stress cold/frost were determined.

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