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Comparative analysis of ICE1 and ICE2 gene expression in Arum Korolkovii (Araceae family) under low temperature stress

Aigerim Yeginbay
Department of Biotechnology, Higher School of Chemical Engineering and Biotechnology, M.Auezov South Kazakhstan University, Shymkent 160000, Kazakhstan
Email: aigerim_eginbai@mail.ru

Altunbek Burabaev
South clinical & Genetic Laboratory, South Kazakhstan Medical Academy, Shymkent 160000, Kazakhstan
Email: altunbek1986@mail.ru

Assilbek Burabaev
South clinical & Genetic Laboratory, South Kazakhstan Medical Academy, Shymkent 160000, Kazakhstan
Email: assilbek@mail.ru.

Raikhan Aitkulova
M. Auezov South Kazakhstnan University

Kuttybek Arystanbaev
South clinical & Genetic Laboratory, South Kazakhstan Medical Academy, Shymkent 160000, Kazakhstan
Email: 201ukgu@mail.ru

Dauylbay Amina
Department of Biotechnology, Higher School of Chemical Engineering and Biotechnology, M.Auezov South Kazakhstan University, Shymkent 160000, Kazakhstan; amina.dd@mail.ru

Seitmagzimova Galina
Chemical Technology Department, M.Auezov South Kazakhstan University, Shymkent 160000, Kazakhstan; galinaseit@mail.ru
*Correspondence: aigerim_eginbai@mail.ru

Abstract—The identification of mechanisms of plant resistance and adaptation to adverse environmental factors opens broad prospects for
the development of breeding biotechnology. The subject of the present study was Arum Korolkovii, a rare endemic species in Central Asia, included in the Red Book of Kazakhstan. In the present work a comparative analysis of expression of ICE1 and ICE2 genes involved in abiotic stress response in the studied plant under cold and frost conditions was carried out. Low temperature stress was induced by placing the plants in cold rooms and reducing the temperature to 0...+2°C for seven days (cold stress), followed by temperature reduction to -4...-6°C for five days (freezing). The relative level of gene expression was analysed by classical polymerase chain reaction. Under stress induction, increased expression of both studied genes, with different levels of expression already at the stage of acclimatization, was shown. The genes studied can be resistance markers for finding donors in gene collections.

**Keywords**— Arum Korolkovii; cold tolerance, frost tolerance, gene expression, electrical conductivity, cell membrane stability.

### Introduction

The issue of biodiversity conservation was first internationally negotiated at the 1972 Stockholm UN Conference on Environment. Subsequently, the UN Conference on Environment in Rio de Janeiro in 1992 adopted the International Convention on Biological Diversity. So far more than 180 countries have acceded to it. The Convention’s main message is that the reduction of biodiversity on the planet poses a serious threat to all humanity. Since the planet is effectively shared by all nations, it is up to them to ensure the full reproduction and functioning of biodiversity in their territories (Conserving Biodiversity..., 2015; Büscher et al., 2016; Arora, 2018; Crist et al., 2021).

Global experience shows that the introduction of cultivated plants is an important additional factor in the conservation of biodiversity. Thanks to this, the botanical gardens and arboretums of the world cultivate and propagate several plant species which have disappeared or are almost disappearing from natural habitats.

In Kazakhstan, many plant species are becoming increasingly rare and under threat of extinction, as in many other regions of the world (Flora of Kazakhstan, 1966; Red Book of Kazakhstan, 2014; Ryabushkina, Abugalieva, Turuspekov, 2016)

The relevance of the use of medicinal plants has increased immeasurably in recent decades. The need to summarise the results of floristic, phylogenetic, ethnomedical, pharmacognostic, phytochemical, phytotoxicological, pharmacological and resource studies of regional floras is constantly increasing.

There has been a significant increase in the number of scientific publications devoted to genetic studies concerning plant stresses and characteristics of their genome properties in adaptation to a variety of environmental conditions.
Whole complexes of diverse genes responsible for certain adaptation properties of plants, including to low temperature stress, are being investigated (Xin, Browse, 1998; Zaretskaya et al., 2012; Samarina et al., 2019; Ban et al., 2017; Hao et al., 2018; Megha, Basu, Kav, 2018; Morsy et al., 2005). Important genes such as ICE1 and ICE2 play a significant role in the overall response to temperature change in plant genomes. The expression pattern of these genes has been demonstrated in many plants, including Arabidopsis thaliana, Camellia sinensis tea, Pyrus ussuriensis pear, Vigna radiata mungbean, Saussurea involucrata, and others (Chinnusamy et al., 2003; Fursova, Pogorelko, and Tarasov, 2009; Huang et al., 2015; Chen et al., 2018; Samarina et al., 2019; Ding et al., 2020; Rout et al., 2020; Wu et al., 2021).

Arum Korolkovii belongs to the rare endemic plant species of Kazakhstan, used in homeopathy and in landscape design. The species is poisonous due to its high alkaloid content but is used in folk medicine as a medicinal plant.

The intraspecific variability of this plant, which provides the potential for adaptation at the population level under conditions of environmental transformation, is clearly understudied, especially with informative methods (Shen et al., 2019; Somerville, 1995; Szekely et al., 2008; Zhu et al., 2018). The weak theoretical development of the population approach to the conservation of medicinal plant resources and the lack of experimental data in this area have made research into the genetic resistance of rare plant species to low-temperature environmental influences urgent.

The aim of this study is to evaluate the intensity of the response of Arum Korolkovii, Araceae family, to low-temperature stress through the expressed expression of cold tolerance genes.

**Materials and methods**

The object of the study was artificially grown plants, Arum Korolkovii from the collection of the South Kazakhstan Medical & Genetic Laboratory of the South Kazakhstan Medical Academy (SKMA) (Fig. 1). The plant was germinated from grains in all-purpose soil (pH = 5.0) in 2-3 liters for three months. The total number of seedlings was 37. All studied plants were kept in laboratory conditions at 20±2°C, with a timely irrigation regime and illumination by fluorescent lamps with 36-55 W and an intensity of 1000-3000 lx. For cold- and frost-resistance studies, a certain part of the leaf lamina with an approximate area of 3-5 mm was taken as control samples. In all stages the first (for RNA isolation) and second (for physiological analyses) leaves were selected, separated by the time of their appearance. Plant leaf pieces were placed in special cold chambers with a temperature regulator. Cold and frost stress studies were carried out with a temperature drop to 0 ... +2°C for seven days (cold stress) followed by a temperature drop to -4 ... -6°C for five days (freezing), while maintaining the required light regime. The physiological test for cold tolerance was carried out according to the methodology developed by Xin et al. (1998).
Electrical conductivity of tissues was checked by conductometric method, damage and stability of cell membranes were determined using OHAUS Conductivity Meter ST300C. The relative conductivity (REC, %) was determined using the following formula:

$$\text{REC} = \frac{L_0}{L_1} \times 100,$$

where $L_0$ is the conductivity of deionised water with leaves at the start of the experiment, $L_1$ is the conductivity of water after 2 h after the start of the experiment.

Cell membrane stability (CMI, %) was determined as:

$$\text{CMI} = \frac{(1 - \frac{L_1}{L_2})}{(1 - \frac{C_1}{C_2})} \times 100,$$
where \( L_2 \) is the conductivity of water before and after boiling (solution cooling), \( C_1 \) and \( C_2 \) are the average conductivity of the control before and after boiling respectively (Bajji et al., 2002).

The level of tissue damage was assessed as 100 - CMI. The results of the study were processed using a single-factor analysis of variance.

DNA isolation was carried out using the CTAB method according to Doyle et al. (1987). RNA for gene expression analysis was isolated from biological samples collected every 2 h according to the method of Furtado (2014). The integrity of isolated RNA was checked by electrophoresis in 1% agarose gel. The amount of RNA was determined on a NanoDrop 2000 spectrophotometer by Thermo Scientific (USA).

The first cDNA strand was synthesized using Promega reagents and protocol (GoTaq® 2-Step RT-qPCR System, A6010) in a volume of 20 μl. The resulting cDNA was diluted to a final cDNA concentration of 12.5 ng/μL in solution.

The polymerase chain reaction (PCR) was performed on an Eppendorf Mastercycler ep gradient S and Mastercycler ProS amplifier. The primer sequences were taken from literature sources, so that only cDNA was involved in the reaction; the list of primers is given in Table 1. The primers were synthesized using a DNA/RNA Synthesizer H8 from Germany and an ASM 800 DNA Synthesizer from Russia.

The following program was used for the amplification: 1 cycle 10 min at 95 °C; 40 cycles 15 s at 95 °C, 1 min at 60 °C. The specificity of the amplification was verified by analysis of the products on an agarose gel.

**Table 1. Primers used in the experiment**

<table>
<thead>
<tr>
<th>Gen</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE1</td>
<td>ICE1-1f</td>
<td>5’-CCCATTAAAAACAGCTGATCACA-3’</td>
<td>Kurbidaeva, 2015</td>
</tr>
<tr>
<td>ICE1</td>
<td>ICE1-r</td>
<td>5’-CCAGCAAGCTAGCTAGGTTGAGTT-3’</td>
<td></td>
</tr>
<tr>
<td>ICE2</td>
<td>ICE2-f</td>
<td>5’-TAAAGGCAAACAACCAAGAGTT-3’</td>
<td>Kurbidaeva, 2015</td>
</tr>
<tr>
<td>ICE2</td>
<td>ICE2-r</td>
<td>5’-TAATCACCCTGTGTAACATC-3’</td>
<td></td>
</tr>
</tbody>
</table>

Electrophoresis in 1 or 1.5% agarose gel in the presence of ethidium bromide (0.1mg/ml) was used to separate the amplification products. An electrophoresis chamber for horizontal electrophoresis was used, conducted in TVE buffer (100 mM Tris/HCl pH 8.0; 10 mM EDTA; 2% SDS).

**Research results**

The screening results showed that the cold tolerance gene ICE1 was present in all the studied Arum Korolkovii plants (Table 2). The ICE2 gene was noted in only 18.9% of the total number of plants in the experiment.
Table 2. Indicators of the Arum Korolkovii screening carried out

<table>
<thead>
<tr>
<th></th>
<th>Total number of plants</th>
<th>Number of plants with the ICE1 gene</th>
<th>Number of plants with the ICE2 gene</th>
<th>Number of plants with ICE1 gene expression</th>
<th>Number of plants with ICE2 gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity, pcs.</td>
<td>37</td>
<td>37</td>
<td>7</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>%</td>
<td>100%</td>
<td>100%</td>
<td>18.9%</td>
<td>91.9%</td>
<td>57.1%</td>
</tr>
</tbody>
</table>

As can be seen, the expression values of ICE1 and ICE2 genes were slightly different in the 37 Arum seedlings tested. The ICE1 gene showed an overall expression of 91.9% while ICE2 was markedly lower than expected with an expression of 57.1%. These data indicate that the ICE2 gene in this Arum species is less sensitive and does not react as strongly to the effects of low temperatures as ICE1.

The study showed that cold exposure at temperatures between 0 and +2°C did not result in significant changes in electrical conductivity of leaf tissues. During freezing in the temperature range from -3 to -6°C, the relative level of conductivity increased significantly, and the stability of cell membranes decreased, indicating an increase in electrolyte release from the tissues (Figure 2).

Figure 2: Changes in leaf tissue parameters at different temperature regimes, 1 - relative conductivity, 2 - cell membrane stability

Significant differences were observed in the expression of the ICE1 and ICE2 genes. The ICE1 gene showed strong activity as reflected by its high level of expression (Fig. 3). The ICE2 gene showed no significant differences in the pattern of response to low-temperature and negative temperature exposures.
Figure 3: Results of electrophoresis in agarose gel in the presence of ethidium bromide, M - molecular weight marker, (1-3) ICE2 gene, (4-6) ICE1 gene

The relative expression level of the ICE1 gene increased upon induction of low-temperature stress (Figure 4).

Figure 4: ICE1 gene expression level measured in hours (1-6)

The results indicate that of the two genes in Arum Korolkovii included in the experiment, the ICE1 gene showed a higher level of expression. When stress was induced, in response to cold exposure, ICE1 gene expression increased 1.5-1.8-fold.

Environmental factors have certain quantitative indicators, such as intensity and range of action. The action of a factor is characterised by its amplitude (Figure 5).
According to the tests carried out, the survival rate when the Arum leaves are frozen below -6°C for 4 days is zero. The survival rate of the plant under study after the tests performed was less than 5 days.

Thus, according to our data, Arum Korolkovii does not exhibit properties of enhanced frost tolerance, and ambient temperatures below -6°C can have a detrimental effect on this plant. This Arum species is markedly sensitive to cold stress, probably because of the insignificant expression of the ICE2 gene. A more thorough investigation of the role of this gene in the metabolic process of the plant species under cold stress requires additional observations.

**Discussion**

There is a range of optimum temperatures for each plant species and deviation from this optimum is a severe stress to the organism. The damaging effects of cold stress can be different at low positive temperatures or at temperatures below 0°C.

As the research shows, frost can have a devastating effect on Arum Korolkovii, and the frost tolerance of this plant is generally low. This is even though the cold-resistance gene ICE1 is present in the plant genome and its expression level is high and reaches 100% in response to low-temperature exposure. A second frost-resistance gene, ICE2, the effect of which was studied in this experiment, showed a generally low expression level. The slow and weak response of this gene does not allow it to cope with the negative effects of a sharp drop in temperature (below -6°C).
Comparative analysis of the action of two genes ICE1 and ICE2 by their expression under cold induction and freezing conditions showed their significantly different relevance for the body's response to cold stress. The experiment confirmed that low-temperature stress leads to disruption of cell membrane stability, which should, in turn, lead to an overall change in lipid, protein and enzyme balance of the cell. The activity of the ICE1 gene, as a response, was significantly increased already at the stage of cold acclimatization, i.e., at the first stage of stress induction. These results are consistent with a number of published studies with other plants in which the increased resistance to cold was due to a rapid response to cold stress. Further studies on the complex of diverse genes involved in the resistance to cold will help to verify the findings in the future.

**Conclusion**

The study of Arum Korolkovii has shown increased expression of the ICE1 gene, while the activity of the ICE2 gene was reduced. Resistance of the plant genotype to low-temperature habitat changes is generally characterized by an earlier response of the ICE1 gene to stress. However, only two known genes were analyzed in this work, whereas for general testing of cold tolerance of Arum Korolkovii it is necessary to involve the whole complex of cold tolerance genes. The study of the expression of all known genes in different organs of A. korolkovii plant under variable low-temperature exposure will be continued in subsequent studies.

**References**


