The effect of drought on the expression of stress resistance genes in perspective forms of birch

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Abstract. Drought stress greatly limits distribution of forest-forming species, in particular, birch. The actual task is to identify resistance mechanisms in order to select perspective genotypes for further reproduction. The purpose of this work was to identify drought-tolerant genotypes of silver birch (B. pendula Roth.), downy birch (B. pubescens Ehrh.) and their hybrids based on the analysis of drought tolerance. We studied the expression of genes encoding proteins of metabolic pathways that are activated in response to abiotic stress (phenylpropanoid way) associated with the pathogenesis of proteins (PR1 and PR10), transcription factors (DREB2), and proteins of late embryogenesis (LEA). As a result of the effects of drought, a significant increase in expression was detected for the PAL, PR-1, PR-10, and DREB2 genes in the analyzed samples; at the same time, expression changes were revealed for the LEA8 gene for two out of ten genotypes. Birch samples 29-58 and 233 was selected as most stable showing adaptive response for all genes analyzed. Analyzed genes can be recommended as markers for assessing drought resistance of other woody plant species.

1. Introduction
Plants often affect by adverse conditions during growth. Climatic factors, such as extreme temperatures (heat, cold, freezing), drought (lack of precipitation, dry wind) and increased salinity of soils, are the main abiotic environmental factors that significantly limit plant growth and development. Drought is a widespread problem, including in the Central Black Soil Region. In connection with the growing problem of drying out of tree species and the loss of their productivity, the issue of forest restoration is urgently raised.

Birch is one of the main deciduous forest-forming species in the European part of Russia where it is creating clean or mixed stands, leaving the first tier. But birch reaches only 20 m in height and often becomes an oppressed breed in competition with spruce or pine. Exposure to abiotic stress, such as drought, is also a significant limiting factor for this breed.

The influence of abiotic factors such as drought, salinity, high and low temperatures can cause significant physiological changes and provoke the development of osmotic stress and loss of cellular turgor. Cell damage including membrane disorganisation, loss of activity or denaturation of enzymes causes ROS (reactive oxygen species) accumulation and leads to inhibition of photosynthesis, metabolic and growth disorders, decreased fertility and premature aging [1, 2].
The need to adapt to changing environmental conditions forced plants to develop a complex defense mechanism that manifests at all levels of the organization. Higher plants are characterized by an active way of adaptation including enhanced growth of the root system, increased water retention capacity, and stomata closing [2]. The response at the cell level includes a change in the membrane system, a modification of the architecture of the cell wall, and changes in the cell cycle and cell division. Physiological and biochemical stress responses to dehydration caused by drought or salinization include repression of cell growth and photosynthesis and activation of respiration. A change in redox metabolism leads to a decrease in ROS and a reduction in cellular redox balance [3-5].

The biochemical response includes osmotic regulation, which consists in the accumulation of osmolytes such as proline, raffinose and glycine betaine and proteins that are specifically involved in the development of stress tolerance [6]. Free proline is accumulated in cells of higher plants for the salt stress compensation [7], which ensures the maintenance of photosynthesis, osmoregulation, macromolecular protection from damage and a decrease in the acidity of cell cytoplasm [8].

A response to environmental stress at the molecular level includes gene expression changes [6, 8, 9] that is controlled by epigenetic regulation [10, 11]. Many genes were revealed that are induced by abiotic stress, and these genes have been classified into two main groups. One group encodes products that directly protect plant cells from stress, that is, proteins that appear to be functional. These proteins are probably involved in the metabolic response to stress, protecting cells from stress by removing toxic elements, restoring cellular homeostasis, and possibly restoring normal growth patterns. These include water channel proteins, proline, detoxification enzymes, antifreeze proteins, and LEA proteins [6, 12, 13]. Lan et al found that 12 of 53 genes of LEA family proteins in Populus trichocarpa were expressed differently depending on the tissue in which they are located and the type of stress affecting plants [14].

Other group products regulate gene expression and signal transduction in response to abiotic stress. It was shown that various transcriptional regulatory systems are involved in the induction of stress-sensitive genes. DREB2 belongs to the gene family of transcription factors, which act as the main regulator of plant response to stress. The expression of DREB2 gene increases due to stress, thus this gene can be used as a molecular marker to identify drought tolerant plants [13].

It is known that several groups of cis- and trans- factors are involved in transcription, which could be controlled or non-controlled by abscisic acid (ABA). Thus, there are ABA-dependent and ABA-independent regulatory systems that influence on the expression of stress sensitive genes. The same group of genes can be induced by different types of abiotic stress, which indicates the presence of cross paths between signaling mechanisms [15].

Pathogenesis related (PR) proteins play an important role in the natural defense against pests and pathogens, their accumulation is also triggered by abiotic stress, a hypersensitive response, and systemic acquired resistance. PR proteins react with various inducers including salicylic acid, jasmonic acid, siderin and ethylene and thus form an intersection point for a plurality of response networks. The detected PRs have been studied in detail and are currently grouped into 17 families of inducible proteins [16-18]. The classification of PR proteins is based on induced expression in response to infection by pathogens: viruses, bacteria or fungi, as a result of injury or exposure to abiotic stress [19-21].

A large number of modern research works have been devoted to the study of gene expression of protein families; an increase in LEA gene expression in response to drought in different parts of cotton [22] plants, drought and salinity in poplar plants [14], abscisic acid in rice plants [23] has been shown. Differential expression of genes of proteins that make up large families in response to stressful influences indicates their participation in the formation of a stress response in plants and allows them to be used as markers of the development of stress resistance.

Understanding the mechanism of the stress response in cultivated plants will help to facilitate and accelerate the selection of promising genotypes [6]. In this regard, the urgent task is to identify resistance mechanisms in order to select promising genotypes of woody plants for further propagation.
and involvement in the breeding process. The aim of this work was to identify drought-tolerant genotypes of birch and their hybrids based on analysis of the expression of drought tolerance genes.

2. Methods and materials

2.1. Plant material and experimental design
The objects of the study were the test cultures of downy birch (*Betula pubescens* Ehrh.) and silver birch (*Betula pendula* Roth.) at the age of 26 years, growing in the territory of the Semiluksky nursery (Voronezh, European part of the Russian Federation). Test cultures of birch were selected for the study of drought tolerance, because the seed progeny (families) survived after the droughts of 2010, 2013, and, therefore, have an adaptive predisposition to high summer temperatures. The origin of stressed birch samples is shown in Table 1.

| No. | Sample | Inventory number | Genetic origin |
|-----|--------|------------------|----------------|
| 1   | 261    | 11-9             | Mother tree 11-9, C - 2*, op-op |
| 2   | 274    | 11-33            | Mother tree 11-33, Silver birch № 1 (Republic of Belarus), op-op |
| 3   | 29-58  | 34-8             | Mother tree 34-8, B - 12, op-op |
| 4   | 327    | 26-26            | Mother tree 26-26, Hybrid (B - 5 x B. mandschurica) |
| 5   | 30-46  | 33-27            | Mother tree 33-27, B - 12, sp-op |
| 6   | 125v   | 35-4             | Mother tree 35-4, B - 17, sp-op |
| 7   | 233    | 26-27            | Mother tree 26-27, Hybrid (B-4 x B. lenta) |
| 8   | 348    | 7-23             | Mother tree 7-23, C - 32, op-op |
| 9   | 15-1   | 20-17            | Mother tree 20-17, hybrid (B-2 x B, pollen mixture) |
| 10  | 264    | 9-11             | Mother tree 9-11, hybrid (C - 3 x B. papyrifera) |

* Abbreviation. op – open pollination, sp – self-pollination (single inbreeding), B – downy birch, C – silver birch.

Birch leaves for analyze drought tolerance were selected in the third decade of June 2019. According to the data of the Voronezh region weather station, the average precipitation of April - June 2019 was significantly lower than the average annual values of the previous 10 years. In June, the amount of precipitation was 39.6 mm less than the mean annual value. The leaves of plants selected from birch trees at the optimum time for air temperature and precipitation observed in the middle of July were used as control.

2.2. RNA (ribonucleic acid) isolation and analysis
For RNA isolation from birch samples CTAB (cetyltrimethylammonium bromide) method by Chang et al. [24] was used with some modifications. Homogenization of samples was carried out using CTAB-buffer of standard composition; the incubation time of samples at 65 °C was increased to 30 minutes, which facilitated the binding of phenolic components and facilitated the further isolation procedure. After cleaning twice with chloroform and isoamyl alcohol, 12 M lithium chloride (1/2 sample volume) was added to the selected supernatant and incubated for 20 minutes on ice, then centrifuged. The precipitate was dissolved in 300 μl of SDS buffer and an equal volume of chloroform: isoamyl alcohol. After centrifugation, the supernatant was precipitated using ethanol and dissolved in deionized water. RNA samples were stored at -80 °C and used for further manipulations.

A qualitative assessment of total RNA was performed using electrophoresis in 1% agarose gel. RNA concentration was determined on a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, USA) using the Qubit RNA BR (broad range) Assay Kit (Thermo Fisher Scientific, 2019, USA). 198 μl of buffer, 1 μl of intercalating dye and 1 μl of sample were added to the measurement cuvette, incubated in the dark for 2 min, then concentration measurements were performed.
2.3. Arranging reverse transcription
Reverse transcription was performed using a standard kit with MMLV-RH (mouse leukemia virus reverse transcriptase (revertase)) (Diaem, 2019, Russia) using 0.5-1 μg of total RNA.

2.4. Selection of primers for birch resistance genes
The search for stress tolerance genes was carried out using literary sources. Primers for the resistance genes of birch samples were selected on the basis of nucleotide sequences presented in the NCBI (National Center for Biotechnology Information) international database. Stress resistance gene sequences primers were selected in the Primer3 program. These oligonucleotide sequences are presented in table 2.

Table 2. Stress resistance primer sequences.

| No. | Gene | Sequence                  |
|-----|------|---------------------------|
| 1   | PAL  | F: CTGTGGCTGCAACCGGT     |
|     |      | R: TCAAATTTGAGGTCCGAGCCA |
| 2   | PR-10| F: GGCCCGGAACCATTAGAAG   |
|     |      | R: CCACCTCGATCAAGCTGTA   |
| 3   | PR-1 | F: CCTCAAGGCCCACAATGACG  |
|     |      | R: TCTCGTCCACCCATAGCTTC  |
| 4   | LEA8 | F: AATGACTTTTGACATGGGCT  |
|     |      | R: TATCCCAAAGTGCAGAGCCA  |
| 5   | GAPDH| F: CAGCCGAAGATGCTCAATGCA |
|     |      | R: GGCCACTTGTTGCTACCAA   |
| 6   | DREB2| F: AGGCAGAGAACACATGGGGA |
|     |      | R: GAAAGTTGAGGCAGCGGTAA |

2.5. Real-time PCR
The conditions of primer annealing were optimized in a temperature gradient (58-70 °C) on a C1000 thermocycler (Bio-Rad, 2017, USA). Real-time PCR was performed using a standard set of reagents in the presence of SYBR Green I dye (Synthol, 2019, Russia) using CFX96 (Bio-Rad, 2017, USA). The reaction parameters were as follows: 95 °C for 3 minutes, then 45 cycles from the stages 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s, then the final elongation of 72 °C for 2 minutes. The GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as a reference.

2.6. Statistical data processing
The relative level of transcripts was determined using the 2-ΔΔCt method using the CFX (коммерческое название, не расшифровывается) Manager software (Bio-Rad, 2017, USA).

Statistical data processing was carried out using the Statistica program. The relationship between the level of gene expression was identified using the Spearman's correlation coefficient (r). All experiments were performed in triplicate.

3. Results and discussion
The expression of stress resistance genes PAL, DREB2, PR-1, PR-10, LEA8 was carried out in 10 birch samples exposed to drought. Analysis of the relative level of PAL gene transcripts showed an increase in this indicator in the experimental samples of 7 analyzed genotypes (figure 1).
Figure 1. Expression of the PAL gene in various birch genotypes exposed to drought. Gray columns – experimental samples, shaded columns - control samples.

An increase in the expression of the PAL gene promotes the activation of the synthesis of the phenylalanine ammonium lyase enzyme, which catalyzes the first reaction of the phenylpropanoid pathway, which produces precursors of many important secondary metabolites, such as tannins and anthocyanins, which contribute to the formation of plant protection under conditions of abiotic and biotic stress.

Analysis of DREB2 gene expression showed an increase in this indicator in all analyzed birch samples except 348 (figure 2).

Proteins DREB2 belong to the family of transcription factors, with a large number of representatives. Activation of DREB2 gene expression in response to abiotic stress, such as drought and salinization, has been demonstrated in various, including woody (poplar) plants.

The LEA family was originally characterized as a collection of proteins that accumulate in high concentrations in the embryos in the final stages of seed development. These proteins are significantly more hydrated than most globular proteins, which may facilitate their participation in the development of plant defense mechanisms under conditions of abiotic stress. An increase in LEA8 gene expression is shown for samples 29-58 and 15-1 (figure 3).
Earlier, when studying the LEA gene family in Populus trichocarpa, it was found that 60% of genes were expressed in vegetative tissues in the absence of stress. At the same time, 9 genes (including PtLEA8-2/4) increased their expression in response to stress, which indicates their participation in the development of stress resistance [14]. In Betula halophila, a drought-resistant species, LEA genes encoding dehydrins also show differential expression in response to salinity [25].

In our study, it was found that in 8 analyzed genotypes, no changes were observed in LEA8 expression relative to control under the influence of salinity. At the same time, two genotypes, 29-58 and 15-01, increase expression when exposed to drought. The results obtained may indicate a genotypically determined expression of genes of the LEA family.

The expression of pathogenesis resistance genes PR-1 and PR-10 also increased significantly in most of the analyzed samples exposed to drought. The relative level of transcripts of the PR-1 gene of samples increased significantly in samples 233, 261, 264, 274, 348, 29-58 (figure 4), and the PR-10 gene in all samples except 327 and 15-1 (figure 5).

Figure 3. Expression of the LEA8 gene in various birch genotypes exposed to drought. Gray columns – experimental samples, shaded columns – control samples.

Figure 4. Expression of the PR-1 gene in various birch genotypes exposed to drought. Gray columns – experimental samples, shaded columns - control samples.
Figure 5. Expression of the PR-10 gene in various birch genotypes exposed to drought. Gray columns – prototypes, shaded columns - control samples.

The family of pathogenesis resistance proteins (PR proteins) plays an important role in the development of plant defense in response to infection by pathogens. Also, the activation of the synthesis of these proteins was shown in response to the effects of abiotic stress, such as the effects of drought and high concentrations of ozone in woody plants, including birch.

Previously, it was revealed that the expression of genes of the PAL and PR 10 groups increased in response to the effects of drought and ozone on Betula pendula plants, and the expression was higher in resistant than in stress-sensitive birch genotypes [26].

According to the results of the study, it was revealed that genes PAL, DREB2, LEA8, PR-1, PR-10 are expressed in different ways in the studied genotypes. In general, a significant increase in DREB2 gene expression was observed in all birch genotypes, except for 348 (table 3).

Table 3. The means of the difference in the expression of drought resistance genes of birch samples from the experimental and control groups.

| Sample name | Change in expression rate, times |
|-------------|----------------------------------|
|              | PAL  | DREB2 | LEA8 | PR-1 | PR-10 |
| 233          | 17.5 | ↑      | ↑     | ↑     | ↑   |
| 261          | 1.5  | ↑      | ↑     | ↓     | ↑   |
| 264          | 2.2  | ↑      | ↑     | ↑     | ↑   |
| 274          | 3.0  | ↑      | ↑     | ↓     | ↑   |
| 30-46        | 6.6  | ↑      | ↑     | ↑     | ↓   |
| 327          | 3.4  | ↑      | ↑     | ↓     | ↓   |
| 348          | 0.7  | ↓      | ↓     | ↓     | ↑   |
| 29-58        | 14.2 | ↑      | ↑     | ↑     | ↑   |
| 125b         | 1.0  | ↑      | ↑     | ↑     | ↑   |
| 15-1         | 0.8  | ↑      | ↑     | ↓     | ↓   |

Arrows ↑ (increase) и ↓ (decrease) show the change of expression of studied samples.

In samples 125v and 15-1, an increase in expression was observed only for the DREB2 gene. Genotype 348 showed an increase in the expression of PR genes, while no response was observed for other groups of genes. Samples of genotypes 233, 261, 264, 274 showed a significant increase in expression for 4 out of 5 analyzed genes (except for LEA8). Samples 29-58 and 233 showed a significant increase in expression for all analyzed genes, which indicates the development of adaptive mechanisms in these genotypes to drought conditions.
The observed significant difference in expression patterns in different birch accessions in response to drought exposure may be associated with the hybrid nature of the accessions that were obtained from different mother trees and have different genetic origins (table 1).

A comparative assessment of the expression of the studied genes showed that there is a significant correlation between the genes DREB2, LEA8, PR-1, PR-10, for which $r_s$ was 0.91-0.98. At the same time, the expression of the PAL gene was characterized by the lowest correlation with the studied genes ($r_s$ did not exceed 0.68).

Exposure to stress, including drought induces a response in plants characterized by multistage and the presence of a wide system of interrelated reactions. Transcription factors are synthesized primarily by activating the synthesis of genes encoding various proteins such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin and so on [27]. The LEA and PR proteins contain DRE elements present in the promoter part, which are targeted for binding by DREB transcription factors that control their expression [27, 28]. Previously, it was found that DREB1 elements regulate the expression of some PR genes under biotic stress [28]. In this study, the presence of clear correlations in the expression of DREB2, LEA, and PR genes may indicate the participation of the DREB2 transcription factor in the regulation of the expression of the LEA8, PR-1, and PR-10 genes by binding to their promoter region.

Data on the evaluation of the expression of stress genes in 10 studied birch samples indicate the development of adaptation mechanisms under conditions of drought. At the same time, all 5 stress markers revealed the presence of adaptive mechanisms in the studied samples of promising birch genotypes; therefore, they can be recommended for stress resistance analysis of various woody plant species.

Significant adaptive ability of genotypes 29-58 and 233, revealed on the basis of analysis of the expression of 5 drought resistance genes, allows us to recommend these samples for introduction into in vitro culture for the purpose of clonal micropropagation and further use in reforestation and afforestation in the forest-steppe zone.

4. Conclusion
As a result of the effects of drought, a significant increase in expression was detected for the PAL, PR-1, PR-10, and DREB2 genes in breeding-valuable hybrids of downy and silver birch; at the same time, expression changes were revealed for the LEA8 gene for two out of ten samples, which indicates the presence of adaptive mechanisms to the effects of drought in the studied genotypes. It was shown that the cascade mechanism of the stress response to drought in the Betula pubescens and Betula pendula hybrids includes the involvement of the DREB2 transcription factor in the regulation of the expression of LEA8, PR-1, PR-10, which specifically binds to the promoter region of these genes.

Samples 29-58 and 233 showed a significant increase in expression for all analyzed genes, which indicates them as the most stable to drought influence and allows them to be recommended for introduction into in vitro culture and mass replication as the most promising. All 5 genes used in the study can be recommended as marker genes for assessing drought resistance and selection of promising genotypes of birch and other woody plants.

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