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ORIGINAL ARTICLE

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The Role of the Methyl Status of Adeniine in the Regulation of the Expression of the Gene of Succinic Semialdehyde Dehydrogenase in Wheat Leaves under Salinity

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Using the real-time polymerase chain reaction method, a change in the level of relative transcription of the *SSADH* gene in wheat leaves under salt stress conditions was established, correlating with changes in activity. A study of the nucleotide composition of the wheat *SSADH* gene showed a certain distribution pattern of GATC sequences in the promoter region, which are methylation sites for adenine DNA methyltransferase. Their content is quite high, which may indicate regulation of the expression of this gene by changing the degree of their methylation. Based on the analysis of the nucleotide sequence of this promoter, primers were developed for its amplification and analysis of the adenylate methyl status. A change in the methylation status of adenines in the GATC sites of the succinic semialdehyde dehydrogenase *SSADH* gene promoter in wheat leaves under salinity was shown.

Key words: succincic acid demialdehyde dehydrogenase, salt stress, regulation, adenine methylation

Salinity is one of the abiotic stress factors that can affect plant organisms (Shihmuradov, 2000). Of particular interest are changes in carbohydrate metabolism, the central link of which is the tricarboxylic acid cycle and the γ-aminobutyric acid shunt. In plants, the GABA shunt is activated under stress conditions, including salinity, and is involved in the adaptation of plants to it by coordinating the carbohydrate and nitrogen metabolism of the plant cell (Li *et al.*, 2021).

Functioning as part of the GABA-shunt enzyme system, the enzyme succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16) metabolizes succinate semialdehyde into the tricarboxylic acid cycle intermediate, succinate (Bouche and Fromm, 1999). Activation of this enzyme during salt stress has been shown by a number of studies (Fedorin *et al.*, 2023, Wang *et al.*, 2023), but the mechanism of changes in activity has been inadequately studied.

DNA methylation plays an important role in the regulation of gene activity at the transcriptional level. Widely studied 5mC methylation usually represses gene expression (Fedorin and Eprintsev, 2022). However, methylated adenine (6mA) marks actively transcribed genes (Zhang *et al.*, 2015), its presence can reduce the energy of DNA duplex separation. Modification of A by methylation can influence interactions with protein factors similar to methyl-CpG-binding proteins, affecting transcription initiation (Fu *et al.*, 2015).

Based on this, the goal of the work was to study the effect of the methyl status of adenine in the promoter of the succinic semialdehyde dehydrogenase gene on its expression in wheat under the influence of salt stress.

MATERIALS AND METHODS

The object of the study was 14-day-old leaves of wheat (Triticum aestivum L.), grown hydroponically. An experiment on the effect of salt stress on plants was carried out by incubating plants of the experimental group (preliminarily deprived of the root system) in a 150 mM aqueous solution of sodium chloride for 24 hours (AbdElgawad *et al.*, 2016).

Total cellular RNA from the leaves of the studied plants was obtained by phenol-chloroform extraction using LiCl as a precipitant (Chomczynski and Sacchi, 1987). The reverse transcription reaction was carried out using the MMLV RT kit (JSC Evrogen, Russia) according to the manufacturer's recommendations.

Real-time PCR was performed on a LightCycler96 device (Roche, Sweden) using Taq polymerase (JSC Evrogen, Russia) according to the manufacturer's recommendations. SYBR Green I was added as an intercalating dye. Amplification was carried out according to the appropriate parameters: preliminary denaturation – 95°C, 5 min; carrying out 45 cycles, including three stages: 95°C – 10 sec; 58oC – 10 sec; 72°C – 10 sec. The final elongation lasted 10 minutes at 72°C. Quantitative matrix control was carried out using the elongation factor gene Ef-1α (Nicot *et al.*, 2005). Calculation of relative transcript levels of the studied genes was carried out using 2-ΔΔCt (Livak and Schmittgen, 2001).

Total DNA from wheat leaves was isolated using 2x CTAB buffer having the following composition: 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-HCl buffer (pH 8.0), 20 mM EDTA and subsequent phenol-chloroform extraction . Isopropanol (98%) and ethanol (95%) were used as precipitant. Agarose gel electrophoresis was used to visualize and analyze the quality of isolated DNA. Nucleic acids were separated on a 1% agarose gel. The gel size was 10×12 cm. The results were visualized using a transilluminator with a wavelength of 312 nm. The results were recorded using the DNA Analyzer photo-video documentation system (DNA technology, Russia) and processed using the Gel Explorer 1.0 program.

Nitrite modification of DNA was carried out by incubating samples for 5 hours at 22°C with the addition of glacial acetic acid, 2M sodium nitrite and proteinase K (Yasaman *et al.*, 2021).

Polymerase chain reaction with primers designed for the promoter of the wheat *SSADH* gene was used using the AmpliSence reagent kit (Helikon, Russia). The PCR reaction was carried out on a Tertsik device (DNA-Technology, Russia) with the following amplification parameters: preliminary denaturation at 95°C for 5 minutes, then 35 cycles: 95°C – 20 s, 62°C – 20 s, 72°C

– 30 s and 72°C – 4 min.

The enzyme Mbo 1 was used to detect the methylated status of DNA at adenine. The pattern of specific hydrolysis of the PCR fragment of the *SSADH* gene promoter from wheat leaves under salt stress conditions was determined by treating 2 µg of DNA using 1 unit of enzyme for 4 hours at 37°C (Fedorin *et al.* ., 2023).

RESULTS AND DISCUSSION

As is known, succinic acid semialdehyde dehydrogenase is encoded by a single gene in the wheat genome. The *SSADH* gene (LOC100284047) is localized on chromosome 6A and contains 20 exons, encoding the mitochondrial form of the enzyme.

Using the Primer Blast software, we developed specific primers for the wheat *SSADH* gene mRNA to assess transcriptional activity by real-time PCR (Table 1).

During this study on the effect of salt stress on changes in the relative level of transcripts of the *SSADH* genes in wheat leaves, its differential expression at different times of the experiment was established. The relative level of *SSADH* gene transcripts increases starting from the first hours of the experiment, reaching a maximum value by the 6th hour of incubation, exceeding the control values by almost 5.2 times (Fig. 1). Subsequently, the level of mRNA of the gene under study decreases, but remains at a higher level relative to the control value.

Thus, it has been shown that salt stress regulates the functioning of SSADH in wheat leaves at the level of transcriptional activity of its gene. This type of regulation can be carried out, among other things, by changing the methyl status of the promoter of a given gene, in particular the adenylate methyl status characteristic of a plant organism (Sun *et al.*, 2015).

To assess the methyl status of adenine in the gene promoter of the enzyme under study, its nucleotide sequence was analyzed for the presence of specific adenylate methylation sites. There are 22 CATG and GATC sites found in the *SSADH* gene promoter. In the region covered by the promoters, there are 4 GATC sites and 2 CATG sites. Primers were developed to assess the methyl status of adenine within GATC sites; their characteristics are shown in Table 2.

A study of epigenetic mechanisms regulating the functioning of the wheat *SSADH* gene under salt stress revealed a difference in the degree of adenine methylation in the promoter depending on the number of hours passed from the start of the experiment (Fig. 2). It was found that the site at position 106, obtained using methyl-specific primers, is normally methylated. After three hours, both the number and size of products increase, indicating a change in the methylation status of GATC sites.

The region covered by the promoters contained two GATC sites. If in samples “0” and “1” only one adenyl nucleotide, the one closest to the 3’ end, turned out to be methylated (product size 106 nucleotides), then in subsequent samples the result of restriction analysis with adenine-methyl-specific nuclease Mbo 1 is different. Analysis of the results of methyl-specific restriction analysis indicates methylation of both GATC sites located in the amplification region of methyl-specific primers. This is evidenced by the presence in the electropherogram of restriction fragments of 254 and 356 nucleotides in size, formed during hydrolysis of PCR products (Fig. 3).

Consequently, the results of the methyl-specific restriction analysis of the promoter region of the succinic acid semialdehyde dehydrogenase gene allow us to conclude that the adenylate methyl status of the promoter of this gene changes when wheat plants are exposed to salt stress. At the same time, one methyl-specific GATC site is constantly methylated, both under normal conditions and under salinity conditions.

But another site analyzed in our study, located at position 356 of the methyl-specific PCR product, undergoes methylation when exposed to sodium chloride. This effect manifests itself after 3 hours of exposure of plants to a 150 mM sodium chloride solution, which is the result of activation of adenine DNA methyltransferase under these conditions. After 12 hours of exposure of plants to a 150 mM sodium chloride solution, a decrease in the methyl status of the *SSADH* gene promoter was observed, which is manifested in a decrease in the number of restriction

fragments (Fig. 2).

Changing the methylation pattern for 6mA near transcription start sites (TSS) plays an important role in the physical association of the promoter with RNA polymerase (Liang *et al.*, 2018 , Karanthamalai *et al.*, 2020). DNA methyltransferase is able to transcribe significantly faster when there are more adenine methyl groups in the transcription start site region (Bochtler and Fernandes, 2021). In contrast to cytosine methylation, adenine methylation has transcriptional activation effects (Fu *et al.*, 2015). The effect we discovered of an increase in the adenine methyl status of the promoter of the succinic acid semialdehyde dehydrogenase gene in wheat leaves during an adaptive response to salt stress correlates with previously published data. Changes in the methylation status of the adenines analyzed in this study within the GATC sites of the wheat *SSADH* gene promoter play a key role in the control of its transcriptional activity.

Thus, it can be assumed that the adenine methyl status of the *SSADH* gene promoter in wheat leaves increases under salinity. It follows from this that adenylate methylation plays an important role in regulating the expression of the gene under study, encoding the SSADH enzyme, as an epigenetic factor in the control of DNA transcriptional activity. The methyl status of adenines in the promoter of the analyzed gene increases from the third to the twenty-fourth hour of the experiment, thereby confirming the active participation of the GABA shunt enzyme in the adaptation of the plant organism to stressful conditions.

Table 1: Nucleotide sequences of primers for assessing the expression of the wheat succinic acid semialdehyde dehydrogenase gene

Gene	Primer	Nucleotide sequences
SSADH	Straight	tgcatttgtcaaggctgttc
	Reverse	ccctaccacagttggctcat

Table 2: Characteristics of primers for the methylated promoter of the wheat *SSADH* gene

Gene	Primer	Nucleotide sequences
SSADH	Straight	cacggcacaaagaacgcacc
	Reverse	ctttctccggtttcgcgcgcc

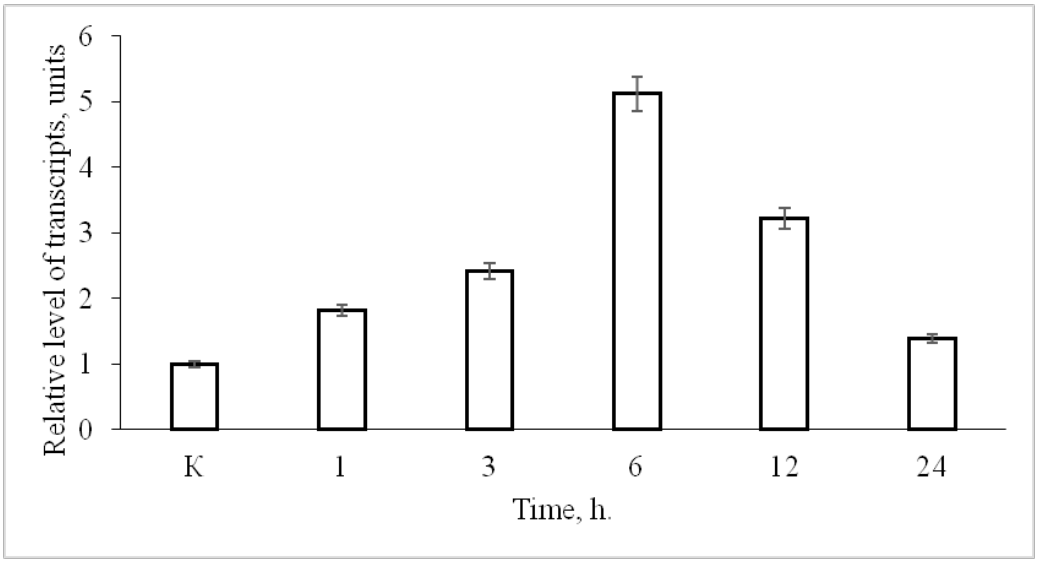

Figure 1. Changes in the relative level of *SSADH* gene transcripts in wheat leaves under salt stress.

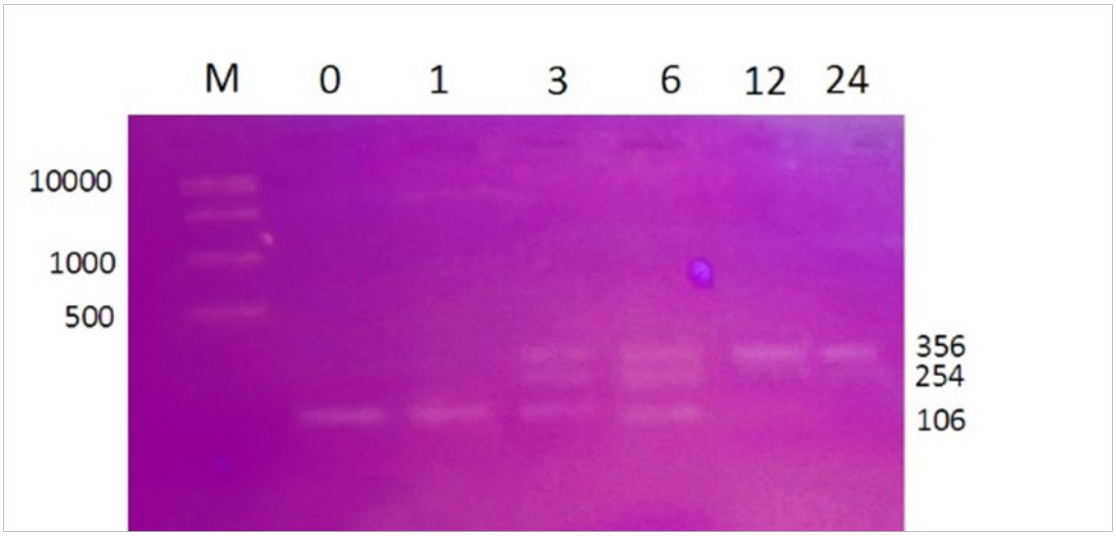

Figure 2. Results of electrophoresis of the products of adenine-specific restriction analysis of the amplicon obtained using methyl-specific primers to the *SSADH* gene promoter.

TCCTTTTAAGCATCATCCACCGACG**GATC**CATGAAGAGTTTAAGGGAGAGCTGTTTTTTCTAGT
GACCATTTGTGGAGGTAGCACAAGTTAGTAGGAGGCACATTTTTCTCTCTCTTCTCCCTCCTTC
CCTCTCCTCTATCCTCTACTTCTTTACCCAATGTAGTGACAGACTTATTTTCTCTCCTCTTCTT
CTTCCTCATCTCTTCTCCTCTACTCTAAACATTCTCCAACGGAGTTGTATGGATGTAATGGGGG
CTGCCCCCAACCCCGTTCA**GATC**TACCCCTGCACCTAACCGAGACATCCAATCACCAGCCGCCC
CACCCATAAATCCACATATCACTTTGTGTTTTGCTATTTCACATCATTAAACAATGTTTTTC

Figure 3. Nucleotide sequence of the PCR product with primers to the wheat *SSADH* gene promoter. GATC sites are in bold; the light gray area is marked with a restriction product 254 bp long, the dark gray area is 106 bp. Both regions together represent a non-restriction digest product whose length is 356 bp.

CONCLUSIONS

The use of the nitrite DNA conversion method made it possible to analyze the methyl status of individual adenines in the promoter region of the *SSADH* gene using adenine-methyl-specific restriction. The use of restriction endonuclease Mbo 1, which specifically hydrolyzes DNA at the GATC site, showed that exposure of wheat plants to a 150 mM sodium chloride solution causes a change in the methyl status of the analyzed adenines in the nucleotide sequence under study.

It has been shown that the transcriptional activity of the gene is regulated at the epigenetic level: the methylation status of adenine in the *SSADH* gene promoter increases over the course of the experiment, correlating with the results of studying changes in the activity and relative level of transcripts of the enzyme succinic semialdehyde dehydrogenase. In this regard, the *SSADH* gene of wheat exposed to salt stress begins to have the ability to transcribe one of the main enzymes of the GABA shunt, which promotes plant adaptation to new conditions.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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