AtDREB2A gene expression under control of the inducible promoter and virus 5'-untranslated regions improves tolerance to salinity in *Nicotiana tabacum*

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Abstract— Transcriptional factor DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A (DREB2A) induces the expression of many genes in dehydration, heat shock, and salinity in *Arabidopsis*. Deletion of sequence coding the 30 amino acid central region transforms full length (FL) protein DREB2A FL into a more stable and constitutively active form known as DREB2A CA. Here, using agrobacteria, a leaf disc transformation of *Nicotiana tabacum* v. Samsun NN was carried out by transgenes *AtDREB2A-FL* and *AtDREB2A-CA* coding the proteins with His-tag on the C-end. The effects of combinations of constitutive 35S CaMV promoter or inducible rd29A promoter with different viral or artificial 5'-untranslated regions (UTR), 5'TMV, 5'PVY, 5'AMV or 5'ARC1, known as translational enhancers were evaluated on the both transgenes' expression. Using an antibody to His-tag, recombinant protein synthesis was detected in transgenic plants in normal and heat shock conditions. After comparative analysis, it was shown that the properties of different 5'-UTRs vary greatly and depended on separate conjunction of promoter and transgene. The integration of *AtDREB2A CA* under control of the rd29A promoter and 5'TMV or 5'AMV in genome effectively improved tolerance of tobacco transgenic plants to 400 mM NaCl and to drought.

Keywords— 5'-untranslated region, translation enhancer, transgene,, transcriptional factor, abiotic stress.

I. INTRODUCTION

For efficient expression of the transgenes all regulatory sequences, including promoters, terminators, enhancers, insulators, silencers and signal sequences are thoroughly selected and detailed examined before stable plant transformation. Preliminary the different expression cassette combinations are constructed until the composition could be optimized to guarantee best results [1]-[3]. These expression cassette uses in transient expression system in leaves or in protoplasts for checking over-expression of recombinant protein and then for stable plant transformation.

For effective gene expression, the transgene is placed under the control of constitutive, inducible or tissue specific promoter, 5'- and 3'-untranslated region (UTR) of viral or synthetic origin. Virus gRNAs are able to compete with plant host mRNAs for the translation apparatus and to switch the plant translation mechanism to preferably synthesis of viral proteins due to the existence of conservative sequences into 5'- and 3'-UTRs or its ability to form specific secondary structure. For example, the gRNA of *Tobacco mosaic virus* (TMV, genus *Tobamovirus*, family *Virgaviridae)* has a 5'-cap structure (m7GpppN), is translated with high efficiency due to the presence of a 5'-leader sequence (5'Ω), which consists several conservative sequences UUAC and $(CAA)_n$ [4]. The expression of the reporter gene under control the 5'Ω could be increased by 2-10 times in the cells of dicotyledonous as well as monocotyledonous plants [5], [6]. An 8-fold increase in the activity of the β-glucuronidase gene was noted when another cap-containing 5'UTR of *Alfalfa mosaic virus* (AMV, family *Bromoviridae*) was used as a 5'-leader in combination with the 35S CaMV promoter [7].

Many viral gRNAs are characterized by a lack of a cap structure. However, instead of this, there are an internal ribosome entry sites (IRES) or cap-independent translational elements (CITE) inside the 5'- or 3'-UTR for effective initiation of translation [8]. In most cases, IRES elements are located within the 5'UTR and/or contain sequences complementary to the 18S rRNA nucleotide sequences, thereby allowing ribosome to take a place on the 5'-end of the gRNA in the immediate vicinity of the initiation codon without scanning the mRNA. A cap-independent translation initiation providing by IRES element inside the 5'UTR is typical for

gRNAs of potyviruses (PVY, *Potato virus Y*; TEV, *Tobacco etch potyvirus*; PPV, *Plum pox virus*; TuMV, *Turnip mosaic virus*) [9]-[11]. For example, the level of translation of synthetic mRNAs increases by 10-24 times in plant protoplasts in the presence of PVY 5'UTR [9], [12], [13].

Many 3'-CITEs found on gRNAs of viruses belonging to the family *Tombusviridae,* such as STNV (*Satellite tobacco necrosis virus*), TCV (*Turnip crinkle virus*) and TBSV (*Tomato bushy stunt tombusvirus*), perform the functions of absent poly(A)-tail [14]-[16]. The 3'-UTRs of these viruses contain sequences are capable to fold into long secondary "stem-loop"-like structures. Due to existence of this structure, the factors of translation initiation are bound with the viral gRNA and delivered this gRNA into the 5'-end. As noted for BYDV (Barley yellow dwarf virus*, Luteoviridae*) and TNV (Tobacco necrosis virus, *Tombusviridae*), there are the over long distance interactions between 3'CITE folded into the secondary structure named as "maple leaf" and 5'-UTR of the same virus which also lead to the binding of initiation factors at the 5'-end of gRNAs and thus facilitate the translation process [17].

The enhancer effect of mentioned 5' and 3'-UTRs has been shown for genes using a wheat cell-free system or as a result of transient expression in protoplasts or leaf disk transformation. But only 5'TMV widely used for stable gene expression in transgenic plants, including genes of transcription factors of DREB (DEHYDRATION-RESPONSIVE ELEMENT BINDING) performing vital regulatory roles in abiotic stress response in plants [18], [19], [20]-[22]. The over-expression of gene coding the one of these factors, DREB2A, in *Arabidopsis*, *Rosa chinensis* and several other transgenic plants such as maize, soybean and peanut resulted in improved stress tolerance [23]-[27].

DREB2A is one of the key transcriptional factors (TF) that is expressed in the response to drought, heat shock, dehydration, and soil salinity [18], [19], [23]. This TF belongs to the AP2/ERF (APETALA2/ethylene-responsive element binding factor) family. Activation of the expression of target genes by DREB2A occurs by a pathway independent of abscisic acid. DREB2A are able to specifically bind through the DNA-binding domain to *cis*-acting DRE/CRT (dehydration-responsive element/C-repeat; A/GCCGAC) elements located in the promoter regions of target genes [19]. Amino acid sequence of DREB2A has two signals of nuclear localization at the N-terminus [19], [24] and negative regulatory domain (NRD) of 30 amino acid residues in central region. The NRD of the DREB2A protein is a hydrophilic sequence, serine and threonine rich, and has the PEST signal found in unstable eukaryotic proteins [19]. During proteolysis the deletion of NRD is happened and full-length protein form is converted into the short but more stable constitutively active form DREB2A CA [23], [24].

Transgenic plants expressing the *DREB2A CA* gene from *Arabidopsis thaliana* under control of 35S CaMV promoter exhibit increased tolerance to drought and heat shock compared to control plants but show growth retardation and reduced reproductive performance [18], [19], [23]. In comparison with the constitutive promoter, the use of the inducible promoter of the *rd29A* (rd, responsive to desiccation) gene for the expression of the *AtDREB2A* transgene, in most cases, does not affect the delay in plant growth and development [19], [23], [28]. After transformation by *DREB2A CA* under control rd29A promoter the transgenic cotton plants were obtained with significant increase in the number of reproductive organs and an improvement in the architecture of the root system in addition to an increase in plant tolerance to drought [20].

If the advisability of using an inducible promoter was shown earlier, there were no reports about comparative effect different 5'-UTR on over-expression of genes of transcriptional factor. Here using the transcriptional factor genes *AtDREB2A-FL* and *AtDREB2A-CA* as an examples, the effect of each four translation enhancers (5'PVY, 5'TMV, 5'AMV and 5'3xARC1 (ARC1, Artificial Regulatory Component)) in combination with the constitutive 35S CaMV promoter or inducible rd29A promoter was compared on expression both forms of the full-length *AtDREB2A FL* and constitutive active *AtDREB2A CA* genes. To distinguish an endogenous protein from a recombinant one, the coding sequences of transgenes were modified for the convenience of subsequent immunodiagnostics. After stable transformation of model plant *Nicotiana tabacum* v. Samsun NN the transgenic lines synthesizing recombinant protein were obtained and tested for abiotic stress resistance.

II. MATERIAL AND METHOD

A. *Plasmid DNA-constructions*

Total RNAs were isolated from the leaves of *Arabidopsis thaliana* v. Colombia or *Nicotiana tabacum* v. Samsun NN using TRI reagent according company recommendations (Sigma-Aldrich, MO USA). Reverse transcription was carried out using 5 µg of total RNAs, 100 pmol oligo(dT)$_{18}$, and kit for Maxima Reverse Transcriptase (M-MuLV RT, Thermo Fisher Scientific, Waltham, MA USA) according company recommendations. The fragments containing the complete nucleotide sequences of the *AtDREB2A FL* gene (1008 bp) and the promoter of the *rd29A* gene (856 bp) were amplified by PCR using *Pwo* polymerase (Roche, Germany) and specific primer pairs (Table I). The primers DR2A-Fw and DR2A-Rv were selected for amplification of *AtDREB2A FL* gene (according to NCBI GenBank acc. no. NM_120623). The primers Rd29A-Fw and Rd29A-Rv were used for amplification sequence of the rd29A promoter (according to NCBI GenBank acc. no. D13044). For both cases the program for PCR was as follows: 94°C, 5 min; 30 cycles at 94°C, 30 s; 58°C, 30 s; and 72°C, 30 s followed by a final stage 72°C, 10 min.

Deletion mutagenesis of the *AtDREB2A FL* was carried out in the pET23d plasmid vector (Novagen) using two pairs of primers, mut-Fw and mut-Rv, DR2A-Fw and DR2A-Rv (Table

I). Deleted *AtDREB2A CA* DNA constructions were selected by the absence of *Bam*HI in 493-499. Primers DR2A-Fw and 8xHis-Rv were used for further mutagenesis of the 3'-end of the *AtDREB2A FL* as described before [29].

A DNA-fragment containing the complete sequence of the rd29A promoter was cloned into pBI221 (NCBI GenBank acc. No. AF502128) between *Hind*III and *Bam*HI instead the 35S CaMV promoter. Cloning of PCR products "*AtDREB2A FL+8His-tag*" and "*AtDREB2A CA+8His-tag"* was carried out between *Nco*I and *Bgl*II into modified plasmid vector contained the 35S CaMV (or rd29A) promoter, 5'UTR, 3'TMV, and *nos*-terminator fused with body of vector pBI221. Then DNA cassettes "*promoter:5'UTR:AtDREB2A: 3'TMV:nos*" were re-cloned into the binary agrobacterial plasmid vector pCAMBIA2300 (NCBI GenBank acc. No. AF234315) between *Hind*III and *Eco*RI.

B. Plant transformation

N. tabacum v. Samsun NN was grown in MS medium supplemented with 30 g/L sucrose and 0.8% agar under standard conditions (25°C, photoperiod 16 h light/8 h dark) in the culture room. The competent cells *Agrobacterium tumerfaciens* strain pGV2260S were electroporated by the recombinant DNA-constructions. Tobacco leaf discs were inoculated by of overnight grown agrobacteria culture diluted to 0.3-0.4 at OD_{600} in liquid MS with a 200 µM acetosyringone. After inoculation during 10 min, the leaf discs were dried on a filter and placed on medium 1 (MS with 1 mg/L BAP, 0.1 mg/L NAA, 20 g/L sucrose, 0.8 g/L agar). After 2 days, the explants were transferred on medium 2 (MS with 20 g/L sucrose, 2.0 mg/L zeatin, 0.2 mg/L IAA, 500 mg/L cefotaxime, 100 mg/L kanamycin, 0.8 g/L agar) and cultivated during 5-6 weeks using fresh medium every 7-10 days. Shoots were cut and put on medium 3 (MS containing 30 g/L sucrose, 500 mg/L cefotaxime, 100 mg/L kanamycin) for root forming.

C. DNA and RNA analysis of transgenic tobacco plants, quantitative RT-PCR

Total DNA was extracted from 100-150 mg leaves by a method using cetyl triethylammonium bromide (CTAB) [30]. PCR was carried out using *Taq*-polymerase Thermo Fisher Scientific kit and GeneAmp 9700PCR System Termocycler (Applied Biosystems, USA). Reaction mix contained 200 µg total DNA, 2.0 µl 10x *Taq*-buffer with $(NH)_4SO_4$, 0.2 µl dNTP (25 mM each), 0.1 µl *Taq*-DNA polymerase (5U/µl), 0.2 µM primers DR2A-527-Fw and 3'TMV-Rv (Table I). Mixture was completed by MilliQ H_2O to a 20 µl total volume. The amplification program was written above. After electrophoresis in 1.2% agarose gel, the fragment in 757 bp size indicated the presence of a full-length gene *AtDREB2A FL*; the band of 667 bp corresponded to the gene *AtDREB2A CA*. The pair primers virD-Fw and virD-Rv (Table I) was used in PCR to prove the absence of agrobacterial infection.

Total RNA was extracted from 60 mg fresh leaves and reverse transcription was carried out as described in section II-A. The primer pair DR2A-527-Fw and 3'TMV-Rv was used for follow PCR.

For real-time quantitative RT-PCR, cDNA was synthesized from total RNA (5 µg) using Thermo Fisher Scientific kit with 100 pmol oligo$(dT)_{18}$ and 200 U Maxima reverse transcriptase in 20 µl volume according manufacture's instruction. Real-time PCR was performed on 7500 real-time PCR-system (Applied Biosystems, USA). Reaction Taq-mix was prepared using a reagent kit from SYNTOL (Russia), 1 µl cDNA, 0.4 pmol each primers (Table I) and SYBR GREEN I fluorescent dye from Applied Biosystems. Amounts of template cDNA that were used in each PCR were corrected by the results of quantification of the tobacco β-actin gene (*act1*) cDNA synthesized with primers Act-Fw2 and Act-Rv2 (Table I). Three determinations were performed for each sample. The analysis of the data obtained was carried out according to the recommendations of Rebrikov et al. [31] in the Q-Gene program.

D. Diagnostics of the recombinant protein

To isolate the nuclei, 1.6-2.0 g of leaves were cut per sample and placed in a Petri dish for 1.5-2 h in normal conditions (24°C) or heat shock (42°C). Percoll (Santa Cruz, TX US) cushions were used according to Ndamukong et al. [32]. The nuclei were resuspended in 500 µl of PBS and treated by TRI reagent for removal of bound nucleic acids. Proteins were resuspended in 60 µl PBS buffer containing 2% SDS and 0.1 mM PMSF.

After electrophoresis in 12% SDS-PAAG, proteins (75 mg) were transferred to a nitrocellulose membrane (Santa Cruz Biotechnology, USA) using semi-dry blotting (C.B.S. Scientific Co). Immunodiagnostics were performed using Penta-His Antibody (5Prime, MD USA) (1:2000) as the primary antibodies and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) (1:2000) as the secondary antibodies. The signal was obtained after treatment with Super Signal West Pico Chemiluminescent Substrate according to the manufacturer's instructions (Thermo Fisher Scientific, IL USA). The membranes were stained by Ponceau S (Sigma-Aldrich, USA).

E. Drought and salt stress conditions

Transgenic tobacco plants were put into the pots 9x9x8 cm in soil. The plants at the stage of 5-7 leaves were used for stress resistance experiments. Three days after the last watering (the beginning of the experiment), the pots were weighed. Then its were incubated without watering in a culture room at a humidity of 30%, 36°C 16 h light /29°C 8 h dark. After 10 days, weighing was carried out every day until a difference in weight reached 1.5-2% per day (about 4-5 days). After that the experiment was stopped and the plants were incubated in the usual regime (24°C, 16 hours of light / 8 hours of darkness). The pictures were taken 1 day before placing the plants in drought and 10 days after resumption of watering. The test was repeated three times. To assess plant tolerance to salinity, small plants at the stage of 3-4 leaves were put on MS medium containing 30 g/L sucrose, 0.8%

agar, and 0 mM, 100 mM, 200 mM, 300 mM, or 400 mM NaCl. After 1 month incubation, the survival rate was assessed by conductometric method. To do this, two leaf cuttings of 1.5 cm in diameter were taken and placed in falcon tubes with 20 ml of sterile bidistilled water. After 3 h incubation on shaker at 24ºC, electrolyte leakage (L1) was determined by measuring the conductivity of solution using a conductivity meter (OK 102/1, Russia). The total amount of electrolytes (L2) was determined after the solutions were autoclaved for 20 min. The electrolyte yield in percent was calculated as: L= (L1/L2) *x* 100 (%).

III. Results

A. Mutagenesis of the AtDREB2A gene

To obtain recombinant DNA-constructs carrying the coding sequence of the constitutively active form *AtDREB2A CA*, deletion mutagenesis was performed in the central region of full-length coding sequence *AtDREB2A FL*. For this aim 90 bp coding 30 amino acid residues from 135 to 165 named as NRD [19] were removed from the position 405 bp to 495 bp. Further mutagenesis of both the *AtDREB2A FL* and *AtDREB2A CA* gene sequences was carried out to generate a sequence encoding eight histidines (8*x*His) on the 3'-end. As a result, the nucleotide sequence gat-ctg-gag-aac coding the amino acid sequence DLEN was replaced by the sequence gat-ggt-gag-cac-cac-cac-cac-cac-cac (8xHis) coding sequence DGEHHHHHHHH at the 3'-end before the stop codons for both genes. Finally, the coding sequences of *"AtDREB2A+8xHis"* (see below as *DREB2A-FL*) and *"AtDREB2A-CA+8xHis"* (see below as *DREB2A-CA*) were cloned into the plasmid vector pCAMBIA2300 under the control of the constitutive 35S CaMV promoter or the inducible rd29A promoter, the one of 5'UTR (5'TMV, 5'PVY, 5'AMV or a synthetic sequence 5'3xARC1 (5'ARC1)), 3'UTR of TMV and *nos*-terminator (Figure 1, Table II). It was previously shown that each of used 5'UTRs had the ability to enhance the level of mRNA translation *in vitro* and *in vivo* [6], [9], [12], [13], [33].

B. Molecular analysis of transgenic tobacco plants

DNA-constructs were used for transformation *N. tabacum* v. Samsun NN. 257 transgenic plants were obtained after regeneration (Table III). Total DNA was extracted from leaves of transgenic plants and was analyzed by PCR to verify the presence of transgene inserts (section II-C). Only 102 PCR-positive transgenic plants were identified, which indicated a transformation frequency of 39.7% (Table III). Although total transformation efficiency wasn't quite different for each transgene, 36.8% for *DREB2A-FL* and 41.2% for *DREB2A-CA*, but this value varied greatly for single DNA-construction. For the full-length gene the transformation efficiency was not dependent on the promoter and on the 5'UTR, because this indicator fluctuated between 30.0% (for *35S:5'ARC1* or *rd29A:5'PVY*) and 45.5% (for *rd29A:5'ARC1*). In contrast, a minimum value of 23.1% was noted when the DNA-construction *35S:5'PVY:DREB2A-CA* was used for transformation. Maximum value of 50% was found out for *35S:5'ARC1: DREB2A-CA* and *rd29A:5'TMV: DREB2A-CA*.

After analysis total plant RNAs by RT-PCR (section II-C), only 35 transgenic lines had the RNA transcripts corresponding to the recombinant cassettes. The most of them (26 lines) were transformed by the *DREB2A-CA* gene (Table IV).

Because some of the obtained plants were transformed by transgene under control of inducible promoter, the presence of recombinant protein examined in 16 transgenic lines under normal conditions 24ºC and after heat shock 42ºC (Table IV). The amino acid sequences of both proteins contained two signals of nucleic localization, therefore nuclear proteins were analyzed. Nuclei were isolated from leaves, then samples treated by TRI-reagent to remove of bound nucleic acids. After electrophoresis, proteins were transferred on nitrocellulose membrane and analyzed by immunodetection with anti-His antibodies. The absence of the proteins expected size about 43-45 kDa was detected for wild type Samsun NN and the line 163 transgenic by vector pCAMBIA2300 as under normal conditions as after heat shock (Figure 2). Finally, recombinant protein was identified in 13 transgenic lines. The protein was detected in plant lines 2, 22, 56, 267 (*rd29A::DREB2A-CA*) 166, 168 and 251 (*35S:5'ARC:DREB2A-CA*) in both cases (Table IV, Figure 2). Although the band was absent in transgenic lines 1, 30, 38 and 263 (lines *rd29A::DREB2A-CA*) in normal conditions, but it was found after heat shock. The recombinant protein wasn't seen in lines 66 and 206 (*DREB2A-FL)* after shock.

C. Drought resistance test

First of all, wild type plants were tested for survival in drought condition for 10, 12, 14, 16, and 18 days. Time period 14-15 days was determined as optimal duration for drought experiments.

The wild-type and the transgenic line 163 carrying the empty vector pCAMBIA2300 were assigned to the negative control group. It was noted that only one of 13 (1/13) plants from this group survived (Table IV). The transgenic lines 1, 2, 17, 22, 38, 56, 66, 206, and 267 were alive after stress, and more than half of these plants continued to grow (Figure 3). All these lines were transformed by the transgenes under the control of the inducible rd29A promoter. Two lines 66 and 206 of all had the full-length *DREB2A-FL* transgene, and another seven lines were transformed by *DREB2A-CA*.

The survival rates of transgenic lines for constructions *35S::DREB2A-FL*, *35S::DREB2A-CA*, *rd29A::DREB2A-FL*, and *rd29A::DREB2A-CA* were found to be 2 of 11 plants (2/11, 18.2%), 2/23 (8.7%), 4/18 (22.2%), and 18/51 (35.3%), respectively (Table IV). The best results were obtained for constructions *rd29A:5'AMV:DREB2A-CA* and *rd29A:5'TMV:DREB2A-CA* (11/14 (78.6%) and 4/7 (57.1%), respectively). In addition, a maximum result of 3/3 was also noted for line 267 of the *rd29A:5'PVY:DREB2A-CA*. None of transgenic lines of the *35S:5'PVY:DREB2A-FL*,

35S:5'PVY:DREB2A-CA, and *rd29A:5'ARC1:DREB2A-CA* were resistant to this stress.

When the content of chlorophyll (a, b, a+b) and the catalytic activity of the catalase and ascorbate peroxidase (data not shown) were compared, no differences were revealed between wild type and transgenic plants either under normal conditions or after drought.

D. Tolerance to increased concentrations of NaCl

After 1 month of incubation, the main effects of NaCl concentrations were found on the control shoots of the wild type *N. tabacum* Samsun NN. Plant phenotype changes were noted at 300 mM of NaCl. Although visually the plants did not look strongly stunted at a concentration of 200 mM and 300 mM NaCl, according to conductometric analysis the plant membranes were damaged even at a concentration of 200 mM and higher. If the electrolyte leakage at concentrations of 0 mM and 100 mM was kept at the level of 46.9 ± 4.7% and 35.8 ± 8%, respectively; then at 200 mM this indicator rose to 58.0 ± 14.4%, at 300 mM to 62.5 ± 17.9%, and at 400 mM to 73.2 ± 12.9% (Figure 4A). In the last two cases, stunted shoots, a lack of normal plant growth, and often a complete absence of root formation (26/39, 66%) was observed due to the damage of the stem base and several lower leaves. The upper leaves became solid and even rigid, deformed, and swollen between the veins with a light green, yellow or light brown color. Shoot growth stopped at concentration of 400-500 mM NaCl, and the lower part of the stem and lower leaves completely died off. The upper leaves looked rudimentary; the growth point was severely deformed. The electrolyte yield in these cases reached 95.2 ± 6.7%.

Although the results of conductometric analysis for the line 163 (vector p[CAMBIA2300) significantly differed from the wild type plants at 100 mM and 200 mM, ion leakage reached the same level as control at 300 mM NaCl, and this indicator increased significantly at 400 mM salt. On the basis of these comparative data, it was concluded that 200-300 mM NaCl were the limiting concentrations for the survival of Samsun NN under stress conditions, and 60-65% were the limiting values of the electrolyte yield.

For constructions *35S::DREB2A-FL*, *35S::DREB2A-CA* and *rd29A::DREB2A-FL* the results were obtained at the same level as the control group. On the contrary, *rd29A::DREB2A-CA* turned out to be the most resistant to 200 mM (electrolyte yield was ~ 50% in contrast to the control - ~ 62%), 300 mM (~ 60% and ~ 70%, respectively), and 400 mM (~ 65% and ~ 87%). However, concentrations of 400 mM and 500 mM damaged membranes in such a way that the plants became stunted and unviable.

Furthermore, a comparative analysis of separate tobacco lines was carried out. Line 113 (*35S:5'ARC1:DREB2A-FL*) showed delayed growth and development both under normal conditions and under stress. The dying-off was noted for the lower leaves at 100-200 mM NaCl. Line 151 (*rd29A:5'ARC1:DREB2A-CA*) was also sensitive to this concentration (Table IV). Growth retardation was typical for most of plants (except three lines 107, 168, and 184) grown at a concentration of 100 mM, if the transgene was under the control of the 35S CaMV promoter, in contrast to the original cultivar and variants under the control of the rd29A promoter (data not shown).

Nineteen lines were more resistant than the control group of plants and the rest of the GM plants (Figure 4A). Four lines (107, 168, 172, and 251) of them were noted with a transgene under the control of a constitutive promoter, and in 15 lines an inducible promoter controlled transgene expression. The electrolyte yield in all these variants was lower than in the control group at 300 mM and 400 mM salt, with the exception of line 193 whose readings approached the threshold value. Although the transgenic lines 1, 22, 30, 38, 263, and 271 looked dwarf in the presence of 300 mM and 400 mM NaCl, the color remained green for most leaves, and shoot growth continued due to weak but stable root formation. All of these plants belonged to the *rd29A::DREB2A-CA* lines and expressed the gene under the control of 5'AMV, 5'TMV or 5'PVY.

To determine the dynamics of changes in the transgene expression, eight transgenic lines with improved tolerance to 300 mM NaCl and higher were analyzed by qRT-PCR (Figure 4B). Under control of constitutive promoter, approximately constant high transgene expression was observed for lines 168 and 251. When the transgene was under the control of an inducible promoter (lines 95, 193, 198, 206, 263, and 271), an increase in the expression level on 100 mM NaCl and a gradual decrease of the expression level on higher salt concentration were detected. In this way, an effect of the plant adaptation to salt stress was confirmed. Only line 206 with full-length DREB2A showed a gradual increase in expression until reaching a maximum value only at 300 mM and a sharp decrease to a minimum level at higher salt concentration.

IV. Discussion

It has been shown for many cellular or synthetic mRNA that viral 5'-NTPs enhance the efficiency of translation several times *in vitro* and *in vivo*, if it's placed upstream of the coding sequence. Such 5'-NTPs allow mRNA to successfully compete with other cellular mRNAs for components of the translational apparatus due to various alternative pathways of translation initiation. However, it has not been investigated whether these advantages extend to mRNAs carrying information about transcription factors, playing a major role in the processes of signal transduction when exposed to unfavorable environmental factors, if their coding sequences are placed under the control of 5'-UTR. In this work the effect of the constitutive 35S CaMV promoter and the inducible promoter of the rd29A gene, and four 5'-UTR (5'PVY, 5'ARC1, 5'AMV or 5'TMV) as the enhancers of various origins and mechanisms of action were investigated on the expression of two transgenes encoding a full-length transcription factor AtDREB2A FL and its constitutively active form AtDREB2A CA in model plant *N. tabacum* v. Samsun NN. Our results

confirmed the fact shown earlier [20], [23], [25] that the constitutively active form *AtDREB2A CA* gene without the sequence coding the negative regulatory domain (NRD) under control of the inducible rd29A promoter was the most suitable transgene for creating plants resistant to abiotic stress. After the tobacco plant transformation 9 and 26 transgenic lines expressing the full-length *DREB2A-FL* and the constitutively active *DREB2A-CA*, respectively, were obtained. Stable transformation efficiency amounted 36.0-45.5% for *DREB2A-FL* and fluctuated over a wider range from 23% to 50% for *DREB2A-CA* (Table III). After drought experiments, the survival rates reached only 18.2% and 22.2% for *DREB2A-FL* under control promoter 35S CaMV or rd29A, respectively. Moreover, after salinity test conductometric values were obtained at the same level as wild type plants. On the contrary, *rd29A::DREB2A-CA* turned out to be the most resistant to 300 mM and 400 mM NaCl showing electrolyte yield values 60% and 65%, that were not exceeded the limiting values. It was concluded that the *DREB2A-FL* expression level and the improved plant tolerance did not depend on the presence of any regulatory sequences, while the type of the promoter or leader was affected the transformation efficiency and the expression of *DREB2A-CA* in plants. This proves once again that the activity of each gene, *DREB2A-FL* or *DREB2A-CA*, was different, and the control for DREB2A-FL protein level was more severe than for DREB2A-CA. Indeed, the level of the endogenous protein DREB2A is very low in normal conditions, and the stress signal is an important event for the stabilization of this factor [24]. The stability of DREB2A is regulated by several different ways. Proteins DRIP 1 and DRIP2 (DREB2A-INTERACTING PROTEINS 1 and 2, homologues of RING E3 ligases) known as negative regulators of DREB2A protein activity promote the processes of ubiquitination and degradation of this protein [34]. Stable accumulation of the GFP protein together with wild-type DREB2A was observed in *drip1 drip2* mutants under standard stress-free conditions, as well as after treatment by an inhibitor of the proteolysis involving the 26S proteasome. In addition, RCD1 (RADICAL-INDUCED CELL DEATH 1) protein interacts with factor through the RCD1-binding site located on C-end of DREB2A, the formed RCD1-DREB2A regulatory unit takes part in negative regulation of DREB2A levels and generally controls abiotic stress responses in *Arabidopsis* [35]. Other proteins, BTB/POZ AND MATH DOMEN (BPM) known as substrate adapters for Cullin3 (CUL3) ubiquitin E3 ligase interact with DREB2A proteins in the nucleus via NRD [22]. Each of the six BPM proteins controls the stability of DREB2A under standard and stressful conditions without affecting on this factor level.

Despite the fact that the level of transcription factors and its stability are regulated by special ways, here the results of the total effect of control by 5'-UTR in conjunction with the regulation of protein activity in plant were studied. For this aim, the DNA-constructs were created in such a way that the sequence around the initiation codon named as Kozak consensus sequence was the same in each case (Table II). The plant lines expressed transgenes under control each of four leaders were obtained (Table IV). Finally, the effect of different 5'-UTR varied in plants (Figures 2 and 4). Transgenic lines 1, 22, 30, 38 and 56 transformed by transgene under control of the rd29A promoter plus 5'TMV or 5'AMV had improved tolerance to drought and salinity. As studied earlier four leaders 5'PVY, 5'ARC1, 5'TMV and 5'AMV had able to enhance of the mRNA translation *in vitro* and *in vivo*, although the dynamics and mechanism of ribosome recruiting for translation initiation differ among these 5'-UTR [5], [6], [7], [12], [13], [33]. Inserting the Ώ sequence of TMV in 5'-UTR of mRNAs gave much more efficient translation in dicot (tobacco) than monocot (oat) cells. The mRNA molecules under control of this UTR remained stable for a long time [5], [6]. The region within the Ώ leader sequence essential for enhancing translation and mapped in position 12-44 nt contains multiple CAA sequences which capable to bind the host heat-shock protein HSP101 in wheat germ extract [4], [36]. Using of 5'TMV as a leader in capped mRNAs enhance translation under eIF4F limiting conditions [37]. But this leader is also capable cap-independent initiation of translation *in vitro*, probably via the internal entry of the ribosomal small subunit to the 5'TMV sequence [38]. In tobacco lines *N. tabacum* v. Xanthi transgenic by 35S CaMV plus 5'Ώ-containing construct GUS-activity observed 37-fold (in leaf), 10-fold (in stem) and 5-fold (in root) higher compared to the construct without a leader [2]. The 5'AMV also has a cap structure at the 5'-end. GUS-activity in tobacco transformed by 35S CaMV plus 5'AMV measured in 48-fold, 4-fold and 11-fold higher, respectively.

As shown our results, these two enhancers have more advantages than 5'PVY and 5'ARC. It turned out that the combination of the 35S CaMV promoter plus 5'PVY negatively affected on the plant viability after transformation. Only three transgenic lines 21, 172, and 173 without any resistant were created for this construction. It was shown earlier in the transient expression system that both 5'PVY and 5'ARC1 enhanced the process of translation initiation due to the presence of IRES elements in their sequences [9], [12], [13], [33]. Both these enhancers had different efficiency and dynamics of action after transient transformation. 5'PVY exhibited "stronger" and more stable prolonged effect at a high level in enhancing of β-glucuronidase gene expression from the beginning than 5'ARC1 with weak effect on translation [29]. Probably, the expression of any transgene under control of the 35S CaMV promoter and 5'PVY was stable at a high level that adversely influenced plant growth and development. It is known that the use of a transgene under the control of the 35S CaMV promoter drives a delay in growth and development and reduces the reproductive system [18], [19], [23]. This effect could be aggravated in case transgene under control of 35S CaMV promoter plus "strong" leader 5'PVY. Only one line 113 (*35S:5'ARC1:DREB2A-FL*) showed growth retardation under normal conditions, but it had "weak" leader.

Thus, the combination of the inducible rd29A promoter and the "strong" leader were preferable for creating plants transformed by *AtDREB2A*. Transgenic lines 95, 263, 271, and 273 with improved tolerance to salinity as well as lines 206 and 267 resistance to drought and salinity belonged to this variant (Figures 2, 3, Table IV).

Although there were not a large number of plants for statistically reliable results to summarize the results of resistance tests, nevertheless, after examination of the general view, the following conclusions were made. The 19 transgenic plants showed an improvement in tolerance to salinity, but only 9 lines of them were more resistant to drought conditions than wild type plants. The impact of the combination "promoter:transgene" was noted for plants undergoing salinity. Even a low level of expression of *AtDREB2A* in lines 95, 198, 206, 263 and 271 was enough to obtain the plant resistance to salinity (Figure 4B, Table IV). Most plants that were able to grow and develop at 300 mM NaCl carried the transgene under the control of the 35S CaMV promoter. While all lines (except for line 168) that stayed alive at the higher salt concentration of 400 mM were transformed by *DREB2A-CA* under the control of rd29A.

The increase in drought tolerance did not depend on as the transgene type as the type of promoter or leader. Although these 9 drought resistance lines were obtained when transgene was under control inducible promoter, two lines 66 and 206 had the insertion of *DREB2A-FL* gene, whereas lines 1, 2, 17, 22, 38, 56 and 267 were modified by *DREB2A-CA*. As for 5'-UTRs, only line 66 had the "weak" leader 5'ARC1, the other 8 plants that successfully passed the drought test were transformed by a transgene under the control of the "strong" leaders 5'PVY, 5'TMV or 5'AMV. And the best results were observed for *rd29A:5'TMV:DREB2A-CA* and *rd29A:5'AMV:DREB2A-CA* (the high survival ratio 4/7 and 11/14, respectively).

Resistance of plants transformed by the same *DREB2A-CA* gene from *Arabidopsis thaliana* to one or another stress was associated with the choice of plants used for gene modification. For example, plant tolerance to drought increased after the integration of *AtDREB2A-CA* into *Arabidopsis*, soybean, sugarcane or cotton genomes [19], [20], [23], [26]. *Rosa chinensis* plants over expressing *AtDREB2A-CA* exhibited tolerance to 300 mM NaCl [25]. The genetic modification of *Arabidopsis* and peanuts by this gene under the control of the 35S CaMV promoter led to an increased tolerance to both drought and salinity [18], [39]. In contrast to the literature, here tobacco plants with improved resistance to both drought and salinity were obtained for both transgenes under the control of the rd29A but not the 35S CaMV promoter. Lines 1, 22, 30, 38, 56 (transgene *DREB2A-CA*) showed good results, as well as lines 66 and 267 (transgene DREB2A) (Table IV, Figures 3 and 4A). These facts confirmed the hypothesis of a common regulatory mechanism for drought- and salt-inducible gene expression [39]. Indeed, in transgenic *Arabidopsis* overexpressed *AtDREB2A CA*, of the total number of genes up-regulated more than 2-fold compared with the wild type, 68 and 107 genes showed drought- and salt-responsive gene expression, respectively [19]. Moreover, 55 genes of 68 genes induced under drought conditions, also responded to salt stress.

Although it is still difficult to obtain direct evidence for the presence of the endogenous protein DREB2A-FL, synthesis of recombinant protein were analyzed in transgenic lines resistant to both stresses. AtDREB2A belongs to proteins of nuclear localization and its small amounts are sufficient for the action of signal transduction of target genes. An endogenous protein in size 43-44 Da was detected by Morimoto et al. [24] in *Arabidopsis* using anti-DREB2A antibodies. In contrast to the constitutively active form, the full length protein is more unstable under normal conditions and is regulated on the posttranslational level by ubiquintin-mediated degradation with the 26S proteasome [34], [39]. According this, it was possible to detect the full-length form protein in normal conditions only using the proteasomal inhibitor MG132 [19], [23], [24]. For this reason, we did not attempt to detect the full-length protein AtDREB2A for all transgenic plants. However, it was shown that it is possible to detect this transcription factor using an alternative method described earlier by Ndamukong et al. [32]. After isolation of the nuclear protein using Percall cushion and the performance of following immunodetection with anti-His antibodies, a product of the expected size of about 43-44 kDa was found only in transgenic lines expressing of the constitutively active form *DREB2A-CA*. The protein DREB2A-CA was found in leaves of transgenic lines 1, 30, 38 and 263 (*rd29A::DREB2A-CA*) only after heat shock; of lines 166, 168 and 251 (*35S:5'ARC1:DREB2A-CA*) as well as lines 2, 22, 56 and 267 (*rd29A::DREB2A-CA*) in both cases in normal and after stress (Figure 2, Table IV). Protein synthesis in first two groups of plants was confirmed by the type of promoter. Transgenes under the control of a constitutive 35S CaMV promoter were expressed in normal and stress conditions, while the use of an inducible rd29A promoter enhanced transgene expression only after stress. In the third group of plants, although the transgene *DREB2A-CA* was under the control of an inducible rd29A promoter, the expression of the transgene also occurs in normal. This could be caused by the position of the transgene insertion into the chromosome, as well as by the effect of expression of the internal gene *NtDREB2A*. In the last case, this transcription factor could interact to DRE-, ABRE- and MYB-binding elements located in the promoter region, inducing the expression of the transgene as a target gene even in stress-absent condition.

V. Conclusion

Expression of genes *AtDREB2A FL* and *AtDREB2A CA* was different in transgenic plants of *N. tabacum* Samsun NN. The combination of a promoter and 5'UTR had affect on the transformation efficiency, expression of the *AtDREB2A* gene, as well as the tolerance of transgenic plants. To create viable

transgenic plants, the combinations “strong promoter: weak enhancer” or “weak promoter:strong enhancer” were the most successful conjunction for the *AtDREB2A CA* gene. The use of the *AtDREB2A CA* gene under the control of the inducible rd29A promoter and cap-containing 5’TMV or 5’AMV was the most preferred for the creation of transgenic tobacco plants with improved tolerance to both stresses. The applied method for the isolation and detection of nuclear proteins could be used for other transcription factors. The further study of the inheritance of the transgene in T1 and T2 generations and stress resistance tests in nature conditions could help to answer the several questions. First of all, whether subsequent recombination events will lead to effective stability or elimination of the transgene? Whether the recombinant protein will be synthesized in next regenerations? Whether the offspring will have resistance to both stresses?

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Oxana Karpova carried out investigation and manuscript writing, developed a methodology.
Alyona Alexandrova created DNA constructions, carried out molecular analysis of transgenic plants, protein analysis.
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Malika Ramazanova was an assistant of plant transformation and stress resistance experiments.
Ruslan Kryldakov carried out optimization and qRT-PCR experiments collect data, statistical analysis.
Bulat Iskakov has organized all experiments, coordinate the work, and write literature review and Discussion.

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Table I. The primers used for cloning of DNA-constructions, transgene diagnostics and qRT-PCR

Primer name	Full nucleotide sequence	Underlined restriction site
Rd29A-Fw	5′-cgtaaagcttcgactcaaaacaaacttacg-3′	*Hind*III
Rd29A-Rv	5′-cgtaggatcctttccaaagattttttcttt-3′	*Bam*HI
DR2A-Fw	5′-gggaaggccatggcagtttatgatcag-3′	*Nco*I
DR2A-Rv	5′-gttttgtttagttgtcgacatccaagtaac-3′	*Sal*I
8xHis-Rv	5′-tacccgggagatcttagtggtggtggtggtggtggtggtg-3′	*Bgl*II
mut1-Fw	5′-cgtcttaatttccct/gatccagattgtgaa-3′	
mut1-Rv	5′-ttcacaatctggatc/agggaaattaagacg-3′	
DR2A-527-Fw	5′-gctgcttctgcttatgatgaggctgctaaa-3′	
3’TMV-Rv	5′-aatccgttatttattatgcatcttgactac-3′	
virD-Fw	5′-gaagaaagccgaaataaagag-3′	
virD-Rv	5′-ttgaacgtatagtcgccgata-3′	
pCAM-Fw7110	5′-cctcgtcctggagttcattc-3′	
pCAM-Rv7809	5′-gcaagtggattgattgatgtgataa-3′	
Act-Fw2	5'-aagcacaataccggtagtacg-3'	
Act-Rv2	5'-ccaaaggccaacagagag-3'	

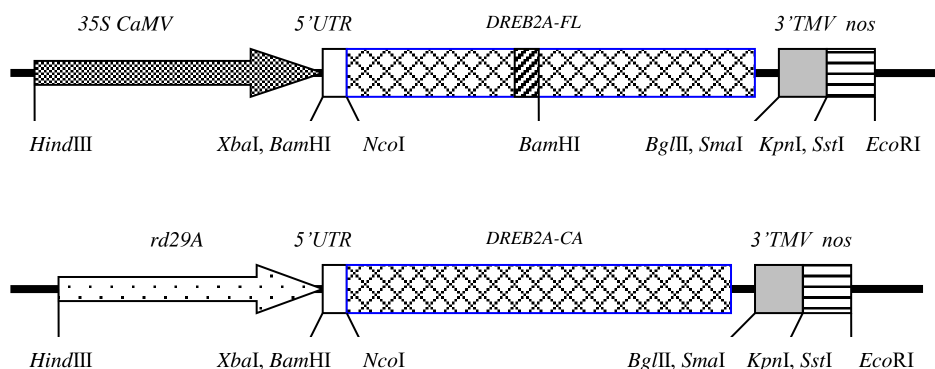


Fig. 1 Schematic representation of DNA-cassettes *«35S:5’UTR:DREB2A-FL»* and *«rd29A:5’UTR:DREB2A-CA»* in the vector pCAMBIA2300. The sequence of constitutive 35S CaMV promoter (upper) or inducible rd29A promoter (down) depicted as arrows. 5’UTR (the one of 5’PVY, 5’TMV, 5’AMV or 5’ARC1) depicted as white rectangle, *DREB2A-FL* and *DREB2A-CA* depicted as rectangles with diagonal rhombuses. 3’TMV marked as grey rectangle, *nos* marked as rectangle with a light horizontal pattern. *DREB2A-FL* contained the negative regulatory domain (depicted dark diagonal pattern upper).

Table II. Complete nucleotide sequences of the different 5’UTRs (the sizes of each was indicated in parentheses, the sequence of the *Nco*I site is underlined)

5’ARC1	5′-ccagcttaca atactccccc acaacagctt acaatactcc cccacaacag cttacaatac tcccccacaa cagcttgtcg acc**atg**g-3′ (83 nt)
5’TMV	5′-ggatccccag ctttattttt acaacaatta ccaacaacaa caaacaacaa acaacattac aattactatt tacaattaca gtcgacc**atg**g-3′ (87 nt)
5’AMV	5′-ggatccaagc ttgtttttat ttttaatttt ctttcaaata cttccacc**atg**g-3′ (48 nt)
5’PVY	5′-aaattaaaac aactcaatac aacataagaa aaacaacgca aaaacactca taaacgctta ttctcactca agcaacttgc taagtttcag tttaaatcat ttccttgcaa ttctcttaaa cgatattgga aaccatttca actcaacaag taatttcatc acttccaacc aattttagat cc**atg**g-3′ (183 nt)

Table III. Efficiency of transformation by transgenes *DREB2A-FL* (full length) or *DREB2A-CA* (constitutive active) under control of different regulatory elements

Cassette name	Full-length *DREB2A-FL* gene		*DREB2A-CA* gene	
	Total number of analyzed plants / Number of transgenic plants	Transformation efficiency (%)	Total number of analyzed plants / Number of transgenic plants	Transformation efficiency (%)
35S:5'Y	27/11	40.7	39/9	**23.1**
35S:5'ARC1	20/6	30.0	24/12	**50.0**
rd29A:5'Y	26/8	30.8	36/17	47.0
rd29A:5'ARC1	22/10	**45.5**	28/13	46.4
rd29A:5'TMV	-/-	-	20/10	**50.0**
rd29A:5'AMV	-/-	-	15/6	40.0
TOTAL:	95/35	36.8	162/67	41.2

Table IV. Transgenic tobacco lines expressing *DREB2A-FL* or *DREB2A-CA*

DNA construction	Transgenic line number	The presence of RNA-transcripts	Expression of protein		Drought tolerance (numbers of plants survived /total numbers of analyzed plants)		Maximal concentration of NaCl (mM), at which plant growth continued
			Normal condition	Heat shock	for the line	for the construction	
Samsun NN	SNN	–	–	–	0/7	0/7	200
Vector (pCAMBIA2300)	163	+	–	–	0/3	1/6	200
rd29A:5'AMV:DREB2A-CA	1	+	–	+	**3/3**	11/14	**400**
	2	+	+	+	**2/3**		nd
	38	+	–	+	**3/5**		**400**
	56	+	+	+	**3/3**		**400**
rd29A:5'TMV:DREB2A-CA	17	+	nd	nd	**2/2**	4/7	nd
	22	+	+	+	**2/3**		**400**
	30	+	–	+	0/2		**400**
35S:5'Y:DREB2A-FL	21	+	nd	nd	0/3	0/3	200
35S:5'Y:DREB2A-CA	172	+	nd	+	0/3	0/6	**300**
	173	+	nd	nd	0/3		200
35S:5'ARC:DREB2A-FL	44	+	nd	nd	0/2	2/8	**300**
	107	+	nd	nd	1/3		**300**
	113	+	nd	nd	1/3		100
35S:5'ARC:DREB2A-CA	161	+	nd	nd	1/2	2/17	200
	166	+	+	+	0/3		200
	168	+	+	+	1/3		**400**
	183	+	–	nd	0/3		**300**
	184	+	nd	nd	0/3		**300**
	251	+	+	+	0/3		**300**
rd29A:5'Y:DREB2A-FL	206	+	nd	–	**2/3**	2/3	**400**
rd29A:5'Y:DREB2A-CA	95	+	nd	nd	0/3	3/12	**300**
	263	+	–	+	0/3		**400**
	267	+	+	+	**3/3**		**400**
	271	+	nd	nd	0/3		**400**
	273	+	nd	nd	nd		**400**
rd29:5'ARC:DREB2A-FL	31	+	nd	nd	0/4	2/15	200
	37	+	nd	nd	0/4		200
	66	+	nd	–	**2/4**		**300**
	236	+	nd	nd	0/3		**300**
rd29:5'ARC:DREB2A-CA	78	+	nd	+	0/3	0/18	200
	151	+	nd	nd	0/3		100
	192	+	nd	nd	0/3		**300**
	193	+	nd	nd	0/3		**400**
	198	+	nd	nd	0/2		**400**
	219	+	nd	nd	0/4		200

Lines with improved tolerance to stress are shown in **bold**, «nd» - not determined, «–» - absence of RNA or recombinant protein, «+» - presence of RNA or recombinant protein.

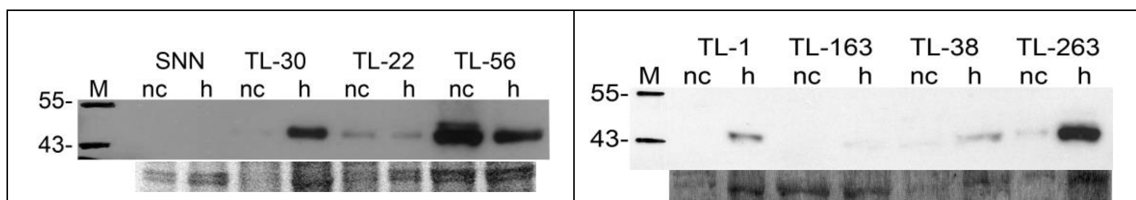
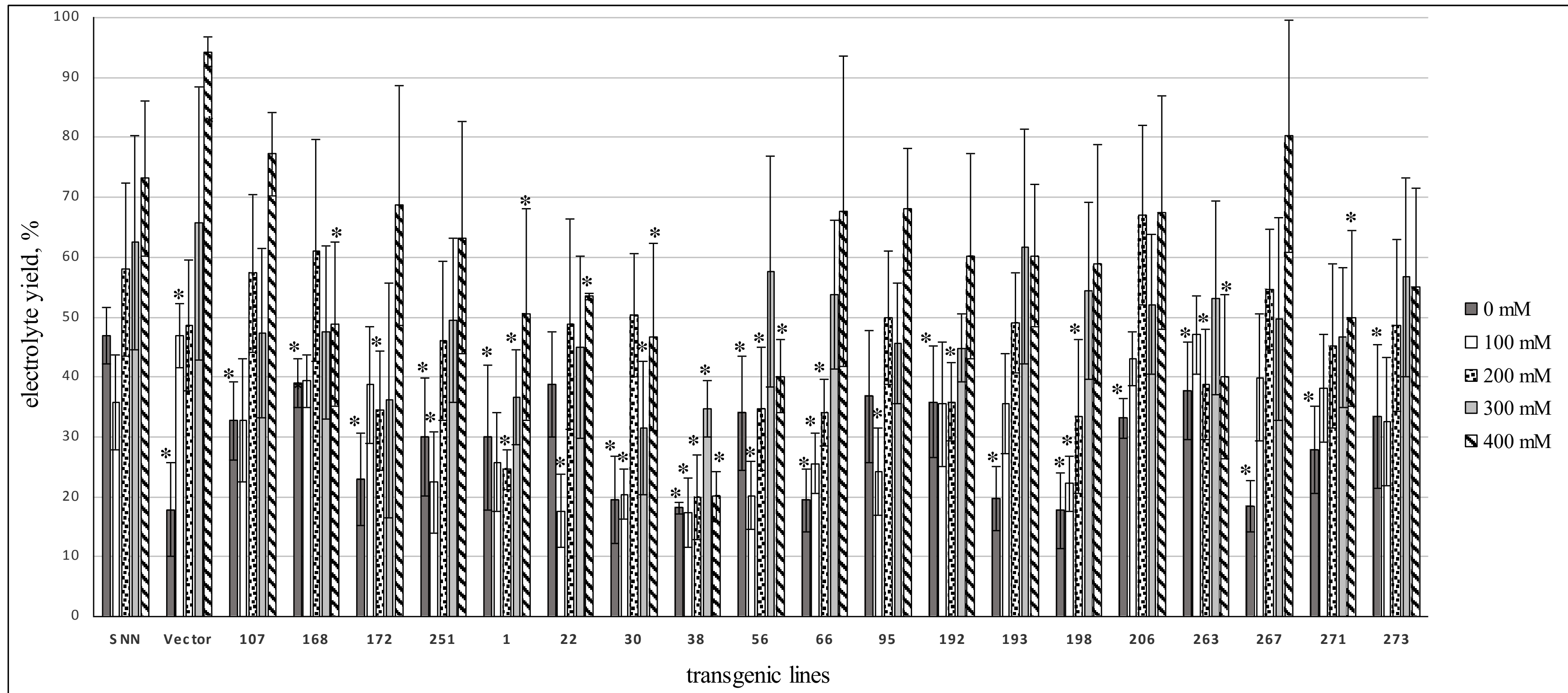


Fig. 2 Detection of nucleic proteins of wild type Samsun NN (SNN), transgenic lines (TL) 1, 22, 30, 38, 56, 263 and line 163 transformed by vector pCAMBIA2300 in normal conditions (nc) and after heat shock (h). M – Markers #26616 ("Thermo Fisher Scientific"). 75 mg of protein were put on each line. The position and size of marker proteins (kDa) was shown on the left. The nitrocellulose membranes colored by Ponceau S were placed in the bottom row.

Fig. 3 Drought resistance test results. a, c, e – the plants before experiments; b, d, f – the plants 10 days after drought experiments. The transgenic lines 31, 95 and 44 (a, b); 206, 107, 173 and 21 (c, d); 1, 267, 184 and SNN (e, f) were shown in upper row. The transgenic lines 66, 161 and 163 (a, b); 56, 78, 151 and 271 (c, d); 168, 113, 30 and SNN (e, f) were shown in down row. The transgenic lines 66 and 161 (b), 206, 107 and 56 (d), 1, 267, 168 and 113 (f) successfully passed drought conditions.

A



B

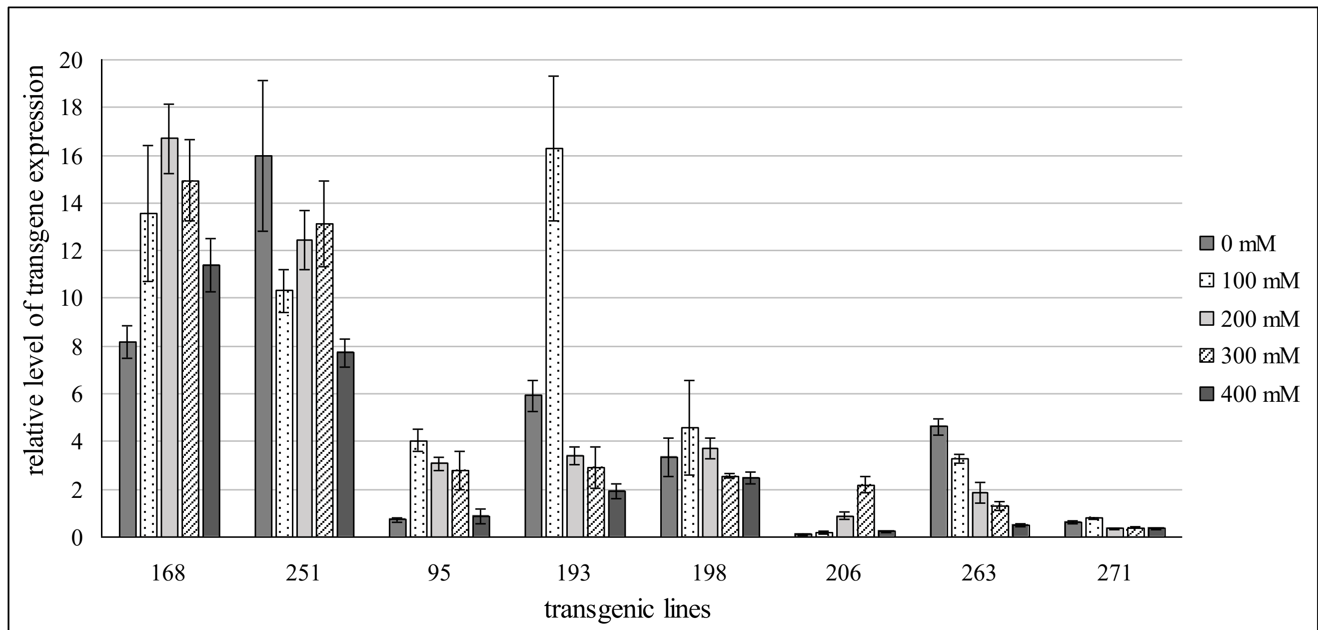


Fig. 4 Stress tolerance to different concentrations of NaCl and gene expression analyses of T0 transgenic lines. A – Leaf electrolyte leakage of the wild type and transgenic lines. Means and standard deviations (SDs) were obtained from three independent experiments. The plants with asterisks were significantly different from wild-type plants (Student's t-test *P<0.05). B – The expression levels of the transgene, estimated by qRT-PCR in transgenic lines grown on MS media with different concentrations of NaCl. The actin gene *act1* was used as a reference gene. The obtained data was processed using Microsoft Excel. Means and SDs were obtained from three independent experiments.