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Characterization of abiotic stress-responsive *RD29B* and *RD17* genes in different poplar clones

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Abstract: Soil salinity as one of the most important abiotic factors limiting productivity becomes a more acute problem worldwide. A significant percentage of land in Vojvodina Province (Northern part of Serbia) are halomorphic soils. Moreover, the process of salinization of land in Vojvodina Province has a tendency to increase in the years to come due to declining irrigation water quality and decreasing precipitations during the years. Consequently, new strategies to enhance yield stability on halomorphic soils become a research priority. Populus species is widely used for afforestation in the Pannonian Plain and due to its sequenced genome it is suitable for genomic analyses of diverse abiotic stresses.
This paper characterizes the abiotic stress-responsive *RD29B* and *RD17* genes in three economically important poplar clones (*Populus* x *euramericana* cl. M1, *Populus deltoides* cl. PE19/66 and *Populus deltoides* cl. 182/81). Understanding the functions of these genes has focused on their responsiveness to salt stress in revealing their expression pattern and mode of induction indicating divergence in potential salt tolerance in poplar clones. Based on the results obtained it was determined that the poplar clones reacted differently to salt stress (150 mM, 300 mM and 450 mM) and showed differential expression pattern of salt responsive genes *RD29B* and *RD17* in each of them respectively. *RD29B* gene expression was elevated in the highly saturated salt conditions and the induction was noticed in the later phases of the stress. *RD29B* gene was selected as a candidate gene for salinity stress breeding of poplar. M1 and PE19/66 clones showed tolerance to higher concentrations of NaCl as a salt stressor and it would be recommended for afforestation of halomorphic environment.

Keywords: *Populus deltoides*, *Populus* x *euramericana*, salt stress, *RD29B, RD17,* transcriptome analyses.

1. Introduction

It is estimated that 20% of all cultivated land is salt-affected, thus reducing yield below the genetic potential (Hasegawa, 2000). A significant percentage of land in Vojvodina Province (Northern part of Serbia) is halomorphic soils. In the Vojvodina region, 2/3 of this kind of soils is in the Banat district. The area of soils affected by salinization is 78000 ha, or 3.6% of the total area of Vojvodina according to Vasin et al. (2010) and Ivanišević et al. (2012). Moreover, the process of soil salinization in Vojvodina has a tendency to increase in the years to come due to declining irrigation water quality and decreasing precipitations during the years. Soil salinity becomes a more acute problem worldwide that

will emerge throughout the years and consequently new strategies to enhance yield stability on halomorphic soils become a research priority (Msane et al. 2011). Populus species are widely used for afforestation in the Pannonian Plain and is extremely important for carbon storage, bioremediation, circulation of biogenic elements, and wood production. In order to afforest salt-affected soils and take advantage of this edaphic deficiency, it is necessary to research in the field of abiotic stress improving salt tolerancy of this valuable forest plant species. Molecular mechanisms of poplar response to abiotic stresses are well studied (Yamaguchi-Shinozaki et al. 1992; Yamaguchi-Shinozaki and Shinozaki, 1993; Brikner et al. 2010; Janz et al. 2010, Ma et al. 2013; Li et al. 2016; Liu et al. 2019).

Analyzing signal transduction pathways between drought stress and gene expression, Yamaguchi-Shinozaki et al. (1992) cloned and characterized nine independent cDNAs named RD (Responsive to Desiccation stress) in *Arabidopsis thaliana*. The research was based on transcriptomic strategies for analyzing the expression patterns of two dehydrin genes, *RD29A* and *RD29B*, that are highly responsive to desiccation stress according to Yamaguchi-Shinozaki and Shinozaki (1993). Two homologous corresponding genes responsive to desiccation stress, named *RD29A* and *RD29B* genes caused attention due to their two-phase induction processes, quick and postponed. Those two homologues appear to be tandem gene duplication encoded very similar proteins. Msanne et al. (2011) stated about a lack of information related to physiological roles and the function of those proteins encoded by two homologous *RD29* genes and found out that *RD29B* homologue was highly induced by salt stress.

Salt tolerant determinant genes can be categorized into two functional groups, one that encodes effector genes responsible for adaptation processes. Those effectors include proteins that protect cells from high cytoplasmic Na^+ such as transporters, biosynthesis enzymes, LEA proteins, chaperones, and detoxification enzymes. The second group consists of regulatory genes that control the expression and activity of the effector genes (Hasegawa et al. 2000).

By studying *A. thaliana* lines constitutively expressing *GmMYB12B2* TF, Li et al. (2016) found out elevated expression level of salt stress-responsive gene *RD17*. This gene belongs to a dehydrin protein family, which contains highly conserved stretches of 7-17 residues that are repetitively scattered in their sequences showing homology with LEA (late embryogenesis abundant) group of proteins. *RD17* contains a DRE or DRE - related motif in its promoter region and is induced by dehydration, salt, and cold stress (Gilmour et al. 1992; Kasuga et al. 1999). This pointed out that *RD17* gene is induced by ABA-independent signal transduction pathway.

Transcriptomic analyses based on salt stress in domestic poplar resources, began recently (Galovic et al. 2015). Extensive research has continued to gather relevant information regarding different salt responsive genes. In this study *RD29B* and *RD17* genes, known as highly responsive genes to salt stress and lacking in functional data for this gene family in woody tree species, were chosen for their functional characterization in three economically important poplar clones. By screening of nucleotide diversity (SNPs) using two homologous GRAS/SCL genes, genetic divergence in the coding regions of those clones were allready proved (Galovic et al. 2015), therefore arise a hypothesis of their different response to salt stressed environment.

By selection of abiotic stress-related candidate genes, *RD29B* (ABA-mediated signal transduction pathway), and *RD17* (ABA-independent signal transduction pathway) and revealing their differential expression during salt stress it will be possible to gather knowledge about the response mechanism of different poplar clones to salt stress environment. Differentially expressed patterns would indicate the nature of salt tolerance dependent background of the clones observed. Acknowledging the pattern and the induction level of the corresponding genes, the tolerance of each clone to grow in severe edaphic environment will be revealed. Furthermore, by overexpressing the most responsive genes in poplar tissue it will be possible to improve the genetic background thus widen the tolerance and adaptive capacity of poplar clones to salt stress.

2. Material and methods

2.1. Plant materials and stress treatments

The study comprised three clones choosen from the Gene pool situated in the Experimental Estate "Kaćka šuma" (45°17' N; 19°53' E) of the Institute of Lowland Forestry and Environment, Novi Sad, Serbia. The clones have different genetic background, where two of them belongs to the species *Populus deltoides* (clones 182/81 and PE19/66) and M1 is a hybrid clone (*Populus* x *euramericana*). The experiment was set up according to a completely random block system. Cuttings (10 cuttings of each clone respectively) were set in Hoagland solution in the aerated conditions of hydroponic culture (Figure 1). After one month, the rooted and proliferated cuttings of poplar clones (*P.* x *euramericana* and *P. deltoides*) were subjected to salt stress.

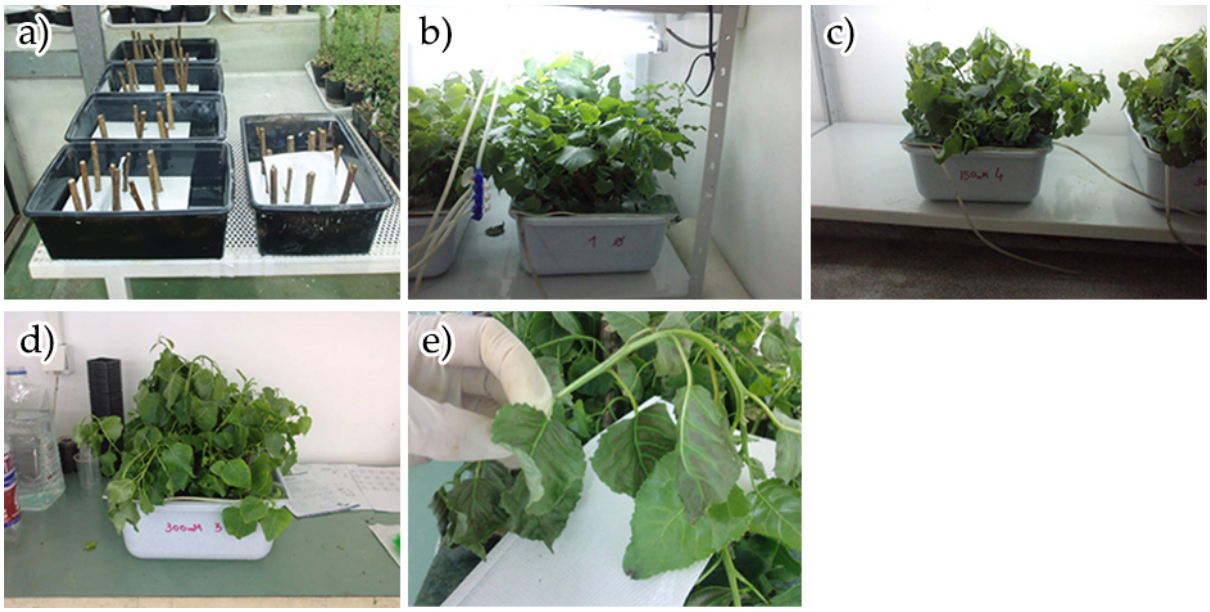

Figure 1. a) Salt stress experiment in the aerated hydroponic culture; b) control plants, 1 months old, rooted and proliferated cuttings; c) 150 mM NaCl stress induction; d) 300 mM NaCl stress induction; e) stress induction: 450 mM concentration of NaCl.

Stress experiment was setted with sodium chloride (NaCl) as a stress agent in concentrations of 150 mM, 300 mM and 450 mM in comparison to control (0 mM NaCl). The system of exposure of plants to salt stress, introducing different concentrations of NaCl to the Hoagland nutrient solution, was gradual from the lowest to the highest concentrations in order to simulate the environmental conditions. Leaf tissue from control and stressed clones were sampled after 3 hours, 8 hours, and 24 hours, liquid nitrogen treated and keep at -80°C until analyzed.

2.2. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The samples were homogenized, total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed, into a more stable complementary DNA (cDNA) biomolecule by the AMV Reverse Transcriptase enzyme (Merck Millipore, https://www.merckmillipore.com). The reaction mixture was prepared by the manufacturer's instruction. Preparations for the qPCR method were performed by checking the RNA quality by electrophoresis, spectrophotometric measurement, and quality. Best dilutions of cDNA were confirmed. Quantitative RT – PCR analysis was performed using SYBR Green JumpStart™ Taq

ReadyMix™ (Merck Millipore, https://www.merckmillipore.com), and a real-time machine using quantitative PCR, Rotor-GeneQ (www.qiagen.com). All PCRs were performed with 12.5 μL of SYBR Green JumpStart Taq ReadyMix, a pair of primers (0.2 μM each), and 10μL of diluted cDNA in a final volume of 25 μL. The qPCR protocol was as follows: 95°C for 10 min and 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. The *β-Actin* gene (AK285936) was chosen as an internal control. Oligonucleotide primer sets were shown in Table 1.

Table 1. Candidate genes (CG), accession numbers and sequences.

Genes	Accession No.		Sequences	Tm (bp)	Product size
PtRD29B	XP_002318815	PtRDB29-F	aaatggttgaggacccagaagt	61.11	139
		PtRDB29-R	tgttccttttgcattgttgttg	60.92	
PtRD17	EEE94764	PtRD17-F	tgaacccgagactaaagtagagga	60.60	118
		PtRD17-R	gtcgctagaagagctggaagaac	61.02	
PaActin7	XM_034932549	PtActin9_F	ggatattcagccccttgtctg	60.90	141
		PtActin9_R	ttctgccccattccaacc	61.00	

Using NCBI database candidate genes were selected to test their expression in stress exposed poplar tissue. Candidate genes, *RD29B* and *RD17*, active in the induction of salt stress were selected. A BLAST search against *Populus trichocarpa* genome using the *Arabidopsis thaliana RD29B*_AT5G52300 and *RD17*_ AT1G20440 sequences was performed. The primers were designed against *Populus trichocarpa*. All preparations for testing expression by quantitative PCR method were performed such as testing quality of cDNA and appropriate dilutions, testing various housekeeping genes. After expression analysis by qPCR (Real-Time PCR) method, samples were analyzed. Statistical processing of data determined the existence or absence of expression for certain genes in the observed clones, as well as the level of expression of certain genes in a certain period. The relative expression level was calculated by normalizing the PCR threshold cycle number of each gene with that of the *β-Actin* reference gene. Based on the obtained results, the molecular mechanisms of the poplar response to salt stress were determined.

3. Results and discussion

To investigate the expression patterns and transcript abundance of *RD29* and *RD17* genes and its response under salt stress conditions quantitative real-time PCR (qRT-PCR) was performed. The *β-Actin* gene (XM_034932549) was chosen as an internal control.

The dehydrin-coding gene, *RD29B*, is usually used as a marker in salt stress. The expression pattern of *RD29B* shows that this gene in M1 clone is activated in the late phases of stress more prominently. Even it was induced in the 150 mM concentration after 24 hours of stress, this gene showed the highest activation in high salt concentration (300 mM) after 24 hours of the stress event. The transcript abundance was somewhat lower in 450 mM but was stable after 3 hours and somewhat higher in 8 hours of the severe stress. Lacking of induction in the first phase of the salt treatment and high expression of a dehydrin gene in the late expression mediated by a high osmotic stress environment it seems that this clone faster recover osmotic homeostasis. Our findings are supported by Brinker et al. (2010) where they revealed decreasing in stress-related genes in the initial salt stress while increases occurred only when leaves had restored the osmotic balance by salt accumulation. They noted that leaves suffered initially from dehydration which resulted in changes in transcript levels of mitochondrial and photosynthetic genes, indicating adjustment of energy metabolism.

Clone 182/81 showed moderate induction of *RD29B* in higher salt concentrations (300 mM and 450 mM) rather than in lower ones. The response was the strongest after 3 hours followed by 24 hours and after 8 hours in the 450 mM. The induction was present but weak also after 24 hours of severe stress. The expression was moderate but stable. There was no induction of *RD29B* gene in 150 mM of

NaCl and in early hours of stress. The activation of *RD29B* is lower in 182/81 than in the two other genotypes.

In the clone PE19/66 the induction of *RD29B* was occurred after 8 hours and the abundance of the transcript was halved after 24 hours in lower concentration (150 mM) of stress agent. It is interesting that this clone had a strong reaction in the early hours of weaker stress and also late, after 24 hours on high concentration like 450 mM of salt stress. Between those activations, there were no induction events of the related gene during the whole experiment. This clone reacts intensively in the starting points of stress and when it is an encounter to the severe salt stress in the medium. There is the occurrence of the 48 hours of the postponed tissue reaction where was no induction of the *RD29B* gene. This doesn't mean that this clone is less tolerant to salt stress but the assumption is more likely that it needs more time for acclimatization to the salt environment. Due to low transcriptional responsiveness of *P. euphratica*, highly tolerant species to high salt stress, Janz et al. (2010) tested the hypothesis of innate expression of stress-protective genes in this species found out that the evolutionary adaptation of *P. euphratica* to salt environments is linked with a higher energy requirement of cellular metabolism and a loss of transcriptional regulation.

It is interesting that the induction of the *RD29B* was not immediate but always later, after 3 hours and later after stress was started considering all concentrations. These results are supported by Yamaguchi et al. (1993a) and Msanne et al. (2011). Those two groups revealed that *RD29B* homolog, a member of LEA dehydrin family, is ABA-dependent and the mechanism of the transcriptional regulation goes on different signal transduction pathways in comparison to *RD29A* homolog that has immediate transcription pattern regarding salt stress.

Clone M1 responded most prominently to severe salt stress by high expression of *RD29B* gene in late activation and the high transcript abundance in the highest concentrations of salt. The transcript level showed high values all the time of the stress duration. Clone 182/81 showed weak expression of the *RD29B* but stable in the higher salt concentrations and in late time-points of the experiment. Clone PE19/66 reacted with two shocking effects in the starting hours and late hours of low and high concentration of salt treatment. Assuming *RD29B* expression pattern in all three genotypes it could be concluded that M1 has the best response to salt stress, recovering osmotic homeostasis very fast also in the severe salt environment following with PE19/66 and 182/81 (Figure 2).

The induction of *RD17* was doubled from 3 to 24 hours after starting of the stress in M1 clone. The transcript abundance was weak throughout the experiment where two peaks was observed after 24 hours of 150 and 300 mM. In severe stress, the response of the *RD17* gene was not significant. The expression of *RD17* was induced in the early hours of the stress till 24 hours of the 300 mM of salt concentration.

Clone 182/81 responded in *RD17* gene induction after 3 hours in 150 mM concentration of salt stress. The transcript level was elevated during the first 24 hours in 150mM almost tri times. Even though there was the same induction rate in 300 mM concentration after 8 hours of stress as in the first hours of the beginning of the experiment, the induction was very strong after 8 hours of severe stress (450 mM). A high transcript level was occurred at the severe environment. The response of this clone was strong and start elevating during the time of the exposure.

The response of PE19/66 to salt stress was in the first hours (3 hours and 8 hours) of stress exposure and mainly in lower concentrations, 150 mM, and with one strong induction in 300 mM. The abundance was moderate. This gene was expressed in this clone but the transcript level wasn't significant. The lowest level of *RD17* gene induction was revealed in M1 clone, then PE19/66, and the most abundant transcripts were notified in the clone 182/81. Findings of Liu et al. (2015) supported our results of elevation of the expression level of *RD17* salt stress-responsive gene. The upregulation of this effector gene (*RD17*) during salt stress can confirm salt tolerancy in related clones. Strong expression of this gene in the clone 182/81 confer its tolerance to salt stress. The regulatory gene, *GmMYB12B2*, that trigger the downstream effector gene *RD17* that contains DRE or DRE-related motif in the promoter region, was not induced by exogenous ABA, indicating that it might be involved in the regulation of gene expression in response to stress through an ABA-independent pathway (Gilmour et al. 1992;

Kasuga et al. 1999). Therefore, *GmMYB12B2* might contribute to basal salt tolerance at least via the activation of the above-mentioned gene.

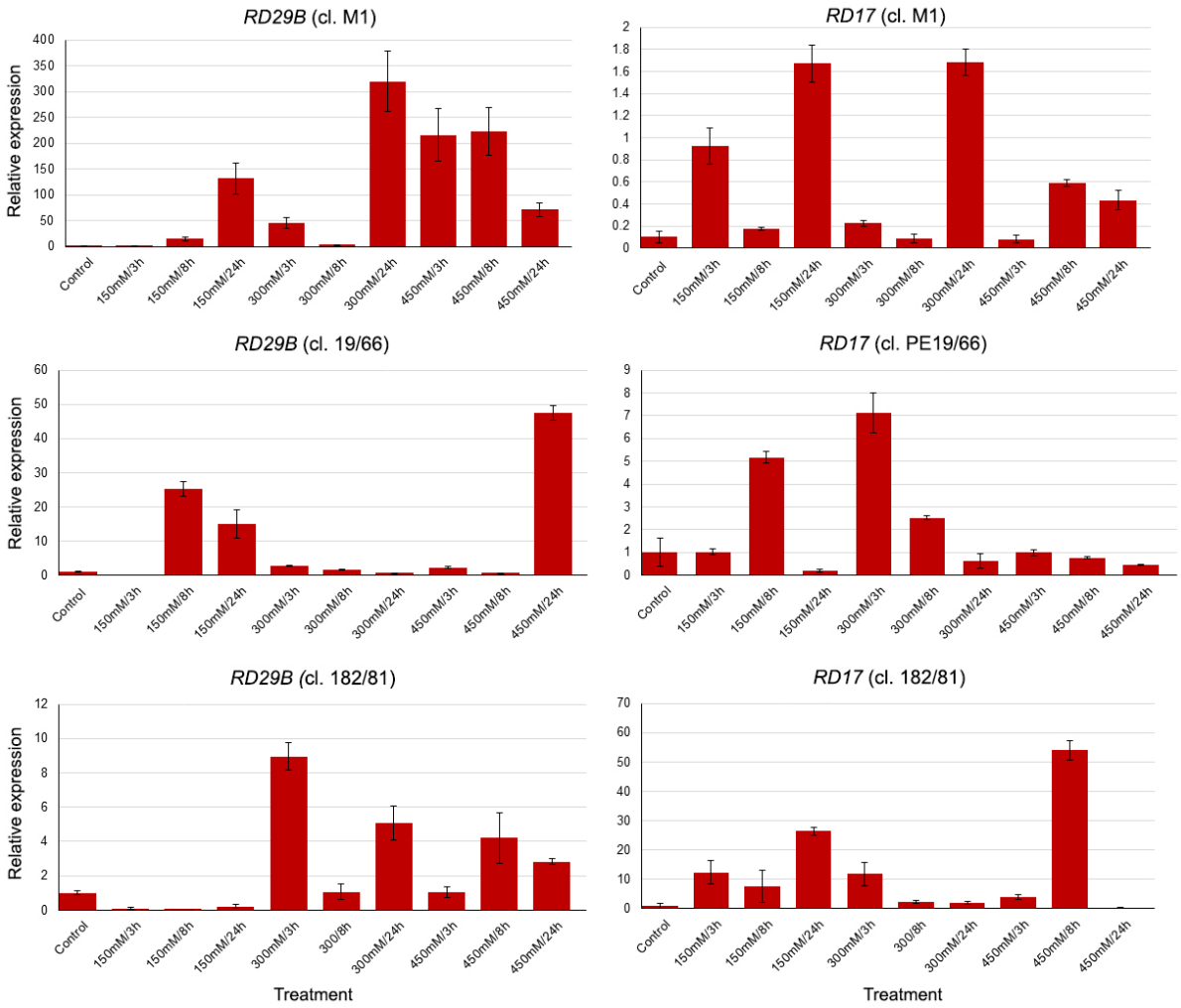

Figure 2. *RD29B* and *RD17* gene expression under different salt concentration (150 mM, 300 mM and 450 mM NaCl) and various time points (3h, 8h, 24h). Transcript levels were determined by Quantitative RT-PCR using oligonucleotide primers that distinguish between the two closely related genes in tissue derived from 3 poplar clones (*P.* x *euramericana* cl. M1, *P. deltoides* cl. PE19/66 and *P. deltoides* cl. 182/81) subjected to salt stress.

Expression of both genes was induced by NaCl stress in all four clones but were differentially expressed in each clone respectively. There was a stronger induction of *RD29B* in comparison to *RD17* in poplar tissue. The pattern of induction was more toward later response for the *RD29B* but earlier for *RD17*. Regarding *RD29B*, the higher transcript abundance was evaluated in clones M1 and PE19/66. During salt exposure, *RD17* gene showed the lowest expression in M1 and in PE19/66 but the highest induction was in the clone 182/81. Since each gene is induced by a different signaling transduction pathway it might be assumed that in the clone M1 and PE19/66 expression of the *RD29B* gene in response to salt stress was regulated throughout an ABA-dependent pathway while in clone 182/81 this regulation goes through ABA-independent pathway expriming *RD17*.

Characterizing two putatively salt stress associated homologues of *GRAS/SCL* TF regulatory gene, Galovic et al. (2015), revealed high nucleotide polymorphisms in the consensus sequences of different conserved parts of the exons of different poplar clones. These findings pointed out their genetic divergence and thus related different potential for salt stress tolerance of each clone. Our results

of species-specific differencial expression of *RD29B* and *RD17* salt responsive genes are in consistency with this study.

Investigating plant mutants defective in ABA biosynthesis, Xiong et al. (2002) were stressed out that they were are more susceptible to environmental stresses. Importantly, manipulating ABA levels by changing the expression of key ABA biosynthetic genes provides an effective means to increase plant stress resistance.

According to the results obtained in this study and all discussion, clones M1 and PE19/66 that highly expressed salt responsive *RD29B* gene in the highly saturated salt stressor, ABA-dependent regulated, would be a recommendation for afforestation of halomorphic environment. By constitutively overexpressing such a gene it will be possible to enhance even more the tolerance of poplar clones to severe salt stress.

4. Conclusion

Based on the results obtained after expression analysis, it was determined that the poplar clones reacted differently to salt stress and showed a differential expression pattern of salt responsive genes *RD29B* and *RD17*. During salt exposure, *RD29B* gene was highly expressed in M1 and PE19/66 clones, while *RD17* gene showed the lowest expression in M1 and in PE19/66, but the highest induction was observed in the clone 182/81.

RD29B gene induction was elevated in the highly saturated salt conditions (300 mM and 450 mM) and its expression was noticed in the later phases of the stress. According to Yamaguchi-Shinozaki and Shinozaki (1993) who revealed quick induction processes for *RD29B* gene, our findings were in correspondence and could be a confirmation of ABA-mediated signal transduction pathways between salt stress and the induction of this gene. Regarding this, *RD29B* was selected as a candidate gene for breeding poplar for salinity stress. M1 and PE19/66 clones can tolerate higher concentrations of salt in the soil and it would be recommended for afforestation of halomorphic environment.

The results of this study will enhance the possibility of afforestation of halomorphic soils as well as growing on poor, abandoned lands and consequently increase afforestation in the Vojvodina Province.

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