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The expression of drought responsive element binding protein (*DREB2A)* related gene from pea (*Pisum sativum* L.) as affected by water stress

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Abstract

Protein pea (*Pisum sativum* L.) is an agronomic type of pea introduced in the region of modern Serbia in the early eighties of the last century. In this study, a new variety NS MRAZ developed by pedigree selection in 2011 was used. Two-week-old plants were subjected to drought stress by withholding irrigation for 7 and 10 days, and rehydrated for one day. Stress effects were monitored by determination of relative water content (RWC), lipid peroxidation and accumulation of reactive oxygen species (ROS).We isolated partial cDNA of *Pisum sativum* DREB2A, namely *PsDREB2A* (HM229349), which belongs to the DREB gene family. Bioinformatics analyses showed high similarity with *DREB2A* gene from model legume *Medicago truncataula*. The relationship between the expression profile of *PsDREB2A* gene and water stress was assayed by quantitative real time PCR in pea roots and leaves. According to obtained results, it is evident that loss of water content strongly induced accumulation of ROS and lipid peroxidation in pea plants. The expression of *PsDREB2A* in pea roots increased with water content decrease, reaching maximum after 10 days of dehydration (2 fold higher than in control plants). On the other hand, in the pea leaves, the highest level of expression was observed after 7 days of dehydration (60% higher than in control). Observed tissue-specific expression profile of *PsDREB2A* suggests complex regulation and the role of this transcription factor in pea drought response. In addition, we can conclude that this pea (var." NS MRAZ") is a drought sensitive plant.

Keywords*:* *Pisum sativum*; water stress; DREB2A; ROS; lipid peroxidation.
Abbreviations*:* ABA-abscisic acid; AP2- apetala 2; CRT- C repeat; DCF- dichlorofluorescein; DCFH-DA-2',7'-Dichlorodihydrofluorescein diacetate; DRE- dehydration responsive element; DREB- DRE binding protein; DW-dried weight; ERF-ethylene responsive factors; EREBP- ethylene responsive element binding proteins; LTRE- low-temperature responsive element; MDA-malondialdehyde; PEG- polyethylene glycol; PLP- phospholipase; ROS- reactive oxygen species; RWC- relative water content.

Introduction

Adverse environmental stresses, such as drought, low temperature and soil salinity have a strong influence on agricultural production and sustainability. A major limitation to yield and quality in many crop species is water availability throughout or at critical times during the growing season (Parry et al., 2005; Morison et al., 2008). Depending on the strength of soil water deficit, plants can maintain photosynthesis and turgor for a short period, or stop growing, show decreased photosynthesis and initiate a series of measures that ensure their survival and developmental processes (Charlton et al., 2008). Across the spectrum of mild to severe drought conditions, plants employ a range of specific responses to minimize the water loss or increase the rate of water uptake (Morison et al., 2008). These include regulation of stomatal conductance (Davies et al., 2002; Buckley 2005), maintenance of cell turgor and osmotic adjustment (Zhang et al., 1999). One of the inevitable consequences of drought stress is enhanced production of reactive oxygen species (ROS) in different cellular compartments, namely in the chloroplasts, the peroxisomes and the mitochondria. This enhanced ROS production is however kept under tight control by a versatile and cooperative antioxidant system (de Carvalho 2008). A number of drought stress-induced genes at transcriptional level have been identified in several plant species. The main regulator of abiotic stress mediated gene expression are transcription factors, among which drought responsive element binding proteins (DREBs) play a key role (Nayak et al., 2009). The dehydration-responsive element (DRE) with the core sequence A/GCCGAC was identified as a *cis* acting promoter element that regulates gene expression in response to drought, salt and cold stress in Arabidopsis (Sakuma et al., 2006). So far, in many plant species, including legumes, DREBs were identified. On the other hand, C-repeat (CRT) and low-temperature responsive element (LTRE) were identified in cold inducible genes (Nayak et al., 2009). The DREB transcription factors induce a set of abiotic stress responsive genes; maintain water balance in plant system and finally impart abiotic stress tolerance. The DREB proteins were divided into two classes - DREB1 and DREB2, based on their role in signaling low temperature and dehydration, respectively. These proteins belong to ERF (ethylene

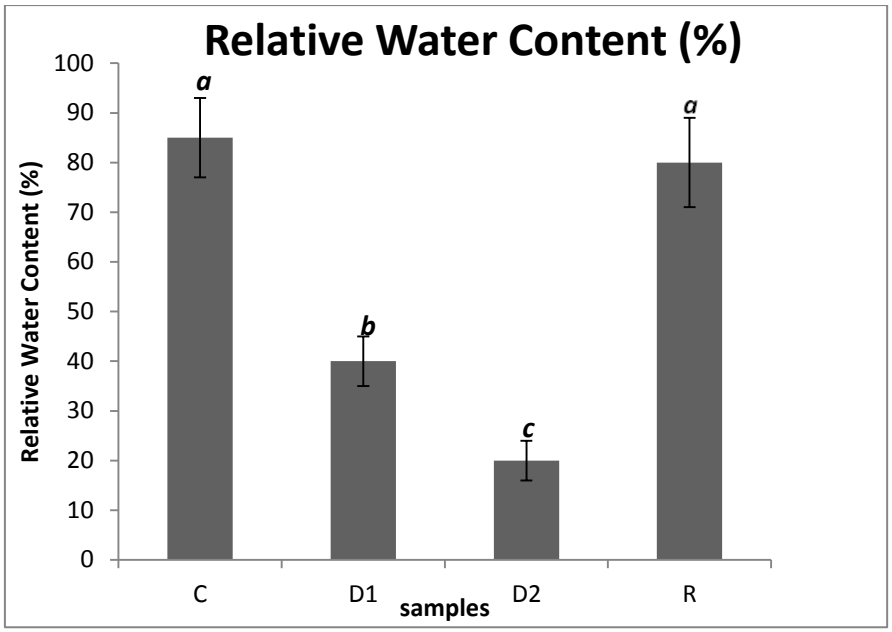


Fig 1. Changes of the relative water content (RWC) in leaves of *Pisum sativum* plants subjected to water stress treatment. C control; D1- dehydrated for 7 days; D2- dehydrated for 10 days;R-rehydrated plants. Data points represent the average of three different measurements ± standard errors based on three replicates. Significant differences ($p \leq 0.05$) between treatments are denoted with different lower case letters.

responsive factors) family of transcription factors. ERF proteins represent a sub-family of the APETALA (AP2) or ethylene responsive element binding proteins (EREBP) that are unique to plants (Shigyo et al., 2006). *DREB1* genes are induced by cold stress and it was found that their over-expression confer tolerance to cold stress in many plant species, e.g. rice, barley, soybean, maize, wheat etc. (Lata and Prasad, 2011; Agarwal et al., 2006). On the other hand, *DREB2A* genes, induced by drought stress, have been reported to confer drought tolerance in several plant species, such as rice, wheat, maize, chickpea, barrel medic, rice and *Arabidopsis*. Despite the fact that both *DREB1* and *DREB2* genes share a sequence similarity at AP2 domain and that they bind to the same *cis* regulatory DRE sequence, the most common evidence is that they are either up-regulated by low temperature (*DREB1*) or by osmotic stress (*DREB2*). However, there are data providing the evidence that this pattern is not obligatory for all plant species. One of very important clues is the existence of post-translational regulation of DREB2 activity. It was shown that such kind of regulation minimally involves stabilization of protein. Under normal conditions, the DREB2A protein expressed at a basal level is degraded via the ubiquitin-proteasome pathway. Upon dehydration and/or heat shock, the protein is further stabilized by some modification (e.g. phosphorilation) or by interaction with other proteins. The function of *DREB1*/*CBF* genes has been well documented in the past decade. However, the function of *DREB2A* was only recently identified by generating a constitutively active form of *DREB2A* (Qin et al., 2008). It was shown that overexpression of the full-length *DREB2A* gene did not result in any remarkable alteration in plant phenotype or the expression of downstream genes. Sakuma et al. (2006) provided the evidence that posttranslational modification was required for the activation of *DREB2A*. Protein pea (*Pisum sativum* L.) is an agronomic type of pea, used in the form of mature grains rich in protein, introduced in the region of modern Serbia in the early eighties of the last century. All the Serbian protein pea cultivars, developed in Novi Sad, are spring-sown and in many seasons are affected by early spring droughts, thus suffering from decreased elaboration of their full genetic potential for high and stable grain yields. A new variety of protein pea cultivar, called "NS-MRAZ", developed by pedigree selection from a hybrid population of various French and Serbian genotypes, was registered in 2011. The aim of this study was to isolate a cDNA clone of dehydration responsive element binding protein (*DREB2A)*-related gene from pea (*Pisum sativum* L.). Based on the sequence information available for the different types of *DREB* genes from model legume species, we designed primer for amplification of putative *DREB2A* from pea. The expression level of that gene was investigated in pea plants subjected to drought stress for 7 and 10 days, as well as in recovered plants. It was shown that dehydration induced the expression of *PsDREB2A* in roots, and that expression profile was different in the pea leaves. This is the first report of this kind, especially having in mind that this cultivar was registered in 2011. In addition, we estimated the level of oxidative stress in pea plants through determination of total ROS level, as well as through the level of lipid peroxidation. This is the first report about the *P. sativum* var.NS MRAZ response to drought stress.

Results

Morpho-physiological changes

During dehydration treatment of pea plants there can be noticed two periods: the initial period of rapid plant dehydration ($C - D_1$) as well as the later period of the slower decline in relative water content, which ultimately led to RWC of 20% in dehydrated leaves (Fig.1). At lower water contents, the leaves underwent some morphological changes, hung down due to loss of turgidity and hereafter curled inward adaxial leaf side, thus reducing the leaf surface exposed to light. At rewatering, plants restored their RWC - after 24 hours of rewatering their relative water content and reached value similar to control (nonstressed) plants (Fig.1).

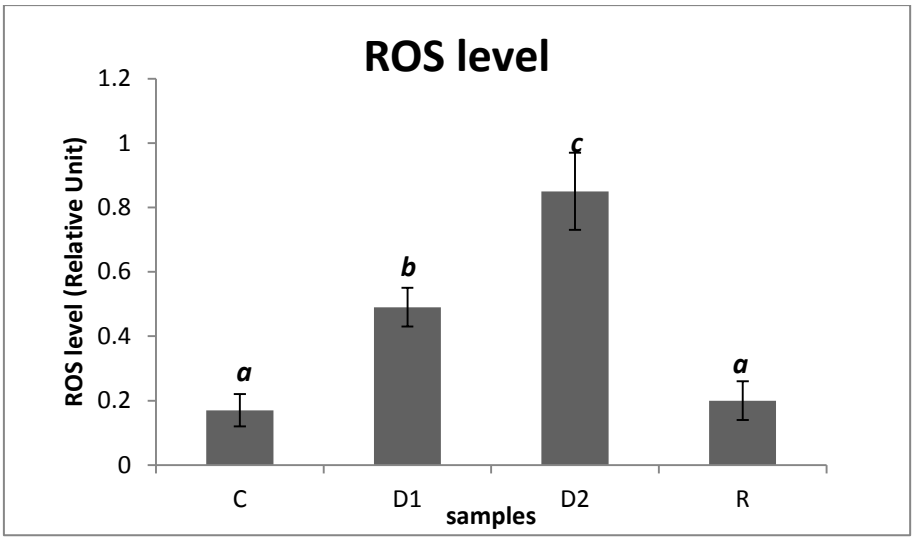


Fig 2. Effect of water stress treatment on ROS level (DCF production rate) in *P.sativum* leaves. C control; D1- dehydrated for 7 days; D2- dehydrated for 10 days;R-rehydrated plants. DCF production rate is expressed as nM min^{-1} per g of dried weight (nM $min^{-1}g^{-1}DW$) ± SE based on three replicates. Significant differences ($p \leq 0.05$) between treatments are denoted with different lower case letters.

Accumulation of total ROS

Dehydration, as well as re-watering of plants caused augmentation of total ROS in leaves, expressed as DCF production rate (Fig.2). In the first dehydration state, after 7 days of treatment, it was evident that pea plants subjected to drought accumulated the reactive oxygen species. The most prominent accumulation of reactive oxygen species was observed in phase 2 of dehydration (D_2), when the level of ROS was more then 5-fold higher than in control, regularly watered plants. After rehydration, the ROS level slightly increased (non statistically significant) in comparison with control plants.

Lipid peroxidation

The level of lipid peroxidation in pea leaves was measured as MDA content, and the effect of dehydration was expressed as the percentage of increase relative to the control (Fig.3). The profile of MDA content was similar to the one observed for determination of total ROS amounts. Initial phase of dehydration (7 day treatment) caused increased level of lipid peroxidation (estimated through MDA content), with the highest level in plants dehydrated for 10 days (D_2). After rehydration, the level of lipid peroxidation was similar to the one observed in control, well hydrated plants.

Isolation of partial DREB2A cDNA from pea

A 493 bp cDNA fragment was isolated from dehydrated leaves of pea (*Pisum sativum*). The corresponding cDNA fragment was named *PsDREB2A* and deposited in GeneBank (HM229349). Nucleotide analysis of obtained sequence showed a high degree of similarity with *DREB2A* gene from model legume *M. truncatula* (DQ908959.1). Further bioinformatics analysis using currently known DREB2-related proteins (against non-redundant protein sequence – nr) showed PsDREB2A sharing a high homology with similar protein from model legume *M. truncatula* (ABJ88942.1) and with ethylene-responsive element binding protein (XP_003616701.1) from the same species. In addition, deduced amino acid sequence revealed a conserved AP2 domain between 17 and 75 amino acids in protein (not shown).

The expression analysis of PsDREB2A

Tissue-specific expression of *PsDREB2A* in pea plants was examined by quantitative real time (QRT)-PCR analysis. As shown in Fig.4a and 4b, the profile of *PsDREB2A* expression was different in roots compared to leaves. In the roots, the water stress treatment induced the increasing of *PsDREB2A* expression, even after first dehydration phase (D_1, 40% RWC). During the prolonged dehydration (D_2, 20% RWC) the expression of *PsDREB2A* reached the highest level - 2.2 fold higher compared to the one in control, well hydrated plants. After re-watering, when plants restored their RWC (similar to control, non stressed plants) the expression of transcription factor was still increased- 60% higher in respect to control plants, but lower than in fully dehydrated plants. On the other hand, the expression of *PsDREB2A* in pea leaves was different compared to the expression observed in the roots. It is evident that the dehydration induced the expression of *PsDREB2A* in pea leaves only during the first dehydration treatment. After 7 days of dehydration, when plants reached 40% RWC, the expression of *PsDREB2A* was at the highest level- 60% higher than in control plants. In plants subjected to prolonged dehydration (for 10 days), as well as in recovered plants, there was no statistically significant change in expression of *PsDREB2A*.

Discussion

It has been demonstrated that many environmental stresses, including drought and salinity, provoke oxidative damage to membranes as a result of accumulated ROS in plant tissues. The determining of the MDA content and thus, the extent of membrane lipid peroxidation, has often been used as a tool to assess the degree of plant sensitivity to oxidative damage (Blokhina et al., 2003). Vendruscolo et al. (2007) reported that MDA content in non-transgenic plants was 65% higher when compared to transgenic plants of wheat during water stress. Our data showed marked increase of MDA content in pea plants which underwent different extent of dehydration.

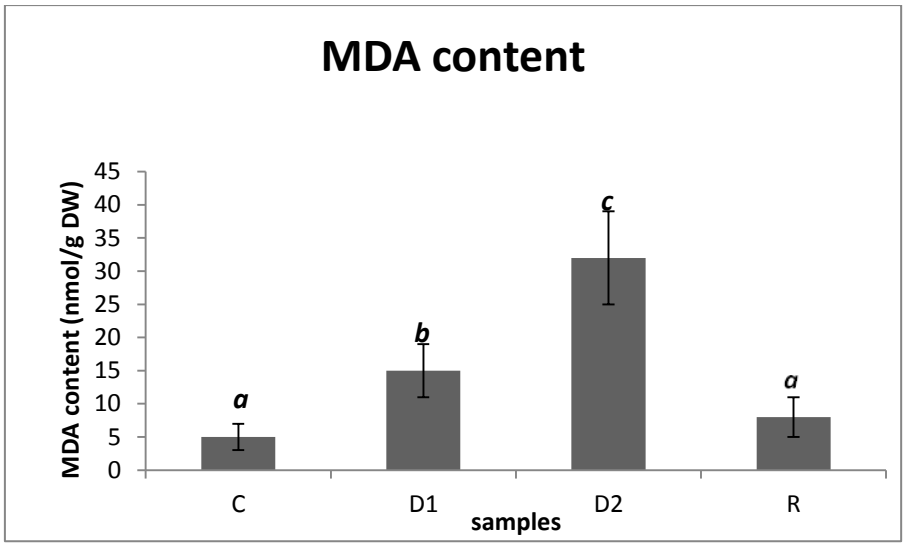
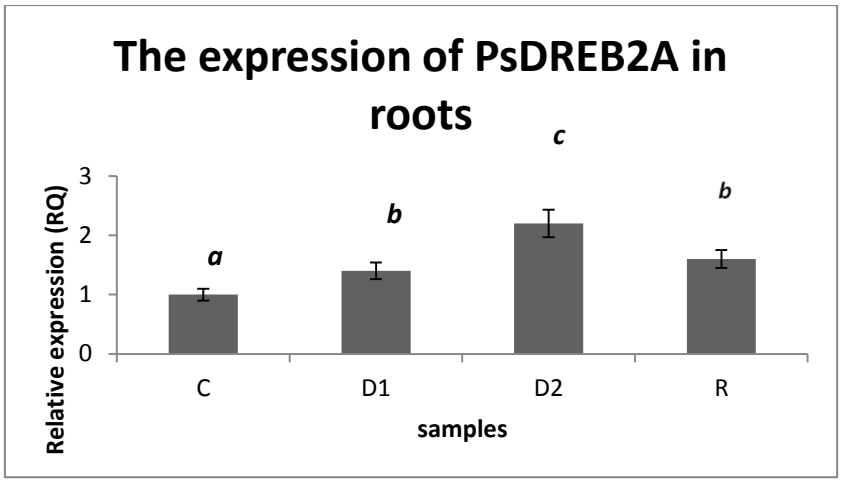


Fig 3. Effect of water stress treatment on MDA content in *P.sativum* leaves. C control; D1- dehydrated for 7 days; D2- dehydrated for 10 days;R-rehydrated plants. MDA content is expressed as nM per g of dried weight (nM g^{-1}DW) ± SE based on three replicates. Significant differences ($p \leq 0.05$) between treatments are denoted with different lower case letters.

The profile of MDA content was similar to the one observed for total ROS level in pea leaves, which is usual. There is a lack of data about pea response to drought stress in field conditions or in a greenhouse. However, a number of experiments describing pea response to drought were performed using different concentration of PEG or by short time dehydration on filter paper. Using our experiment design, most similar to field conditions, according to the accumulation of ROS and subsequent membrane damages (estimated through MDA level) we can assume that pea (var. "MRAZ") is a drought sensitive plant, even though it rehydrated after rewatering. The confirmation for that was the result of Egert and Tevini (2002); they observed that lipid peroxidation remained unchanged in plants tolerant towards salinity or drought. After the discovery of DREB proteins in Arabidopsis and of their role in abiotic stress response, homologue genes were discovered in other plants, including legumes and crops (Nayak et al., 2009). Though the DREB1 class of transcription factors has been studied in some detail, information on DREB2 is limited. Here, we report the isolation and characterization in pea (*Pisum sativum*) of a new member of *DREB*-related gene family, namely *PsDREB2A*. For its isolation and amplification we used available sequence, especially sequence of *DREB2A* from model legume plant *Medicago truncatula*. Despite the fact that pea is a member of tribe Viceae and *M. truncatula* belongs to tribe Trifoliae, we successfully used available sequence for primer design. The pea genome is much larger (~10 times) than that of *M. truncatula* and has a base chromosome number of 7, compared to 8 in *M. truncatula* (Choi et al., 2004). The analysis of 57 gene-specific markers revealed broad conservation of genome structure, with the major evident differences being sites of inferred chromosomal rearrangements. It is known that *DREB2A* gene from *M. truncatula* does not contain intron, and that there are the same PCR products which use genomic DNA or cDNA. We used cDNA from treated, water stressed plants for isolation of *PsDREB2A*. However, nucleotide analysis revealed high level of similarity with *DREB2A* gene from model legume *M. truncatula* (DQ908959.1). A lower similarity was observed with other legume species (soya). There are no data about *DREB2A* genes from other plants from tribe Viceae (lentil and fava bean), and these data can be interesting for phylogenetic studies. BLAST analysis on protein level showed that PsDREB2A shared a high homology with similar protein from model legume *M. truncatula* (ABJ88942.1) and with ethylene-responsive element binding protein (XP_003616701.1) from the same species. Also, the existence of conserved AP2 DNA-binding domain, with other data, definitely assigned that protein to DREB family. There are a lot of data about the role of DREB2A proteins in abiotic stress tolerance in plants. The expression analysis of *PsDREB2A* was determined using quantitative real time PCR. We used plants grown in the greenhouse, and the time-course water stress experiments took 10 days. These conditions probably prevented the evaluation of the expression pattern at earlier stages in the stress response, but we were interested in relating changes in gene expression during a period that reflects as closely as possible the time frame during which water stress starts having visible effect under field conditions. The similar experiment was performed by Latini et al. (2007) when they investigated the expression of *DREB2A* from *Triticum durum*. Also, to avoid the influence of potential circadian clock, we collected all the samples at the same time on the day of collection (10^h). Bieniawska et al. (2008) showed that circadian clock was responsible for extensive variation in the cold-responsive transcriptome. Despite the fact that there are no data about such influence on DREB2A expression, we could not exclude the possibility of the existence of such regulation. Our results showed that dehydration stress strongly induced the expression of *PsDREB2A*, dominantly in root tissues. During the first phase of dehydration (40% RWC), the expression level was 2 fold higher than in control, non-stressed, well-watered plants. After that, in more dehydrated plants, the expression of *PsDREB2A* in roots was at the level similar to the control. However, plant rehydration caused a slight increase in its expression, suggesting that even rehydration could be some kind of stress. On the other hand, in the leaves, the expression of *PsDREB2A* was slightly induced only during the first dehydration treatment, suggesting that the expression of transcription factor had the main role in the roots. This is consistent with a well-known fact that roots act as a first place of drought perception. The observed changes in time-course of expression are not unusual. Experiments performed on chrysanthemum



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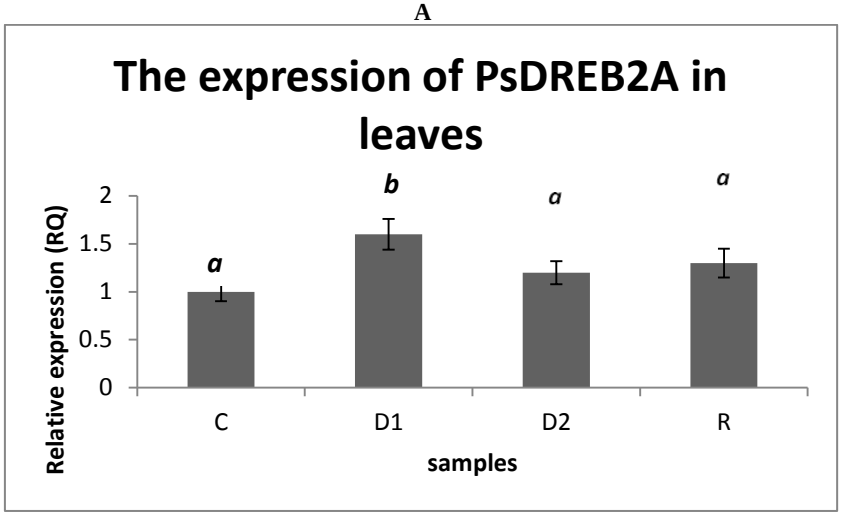

B

Fig 4. Relative expression level of *PsDREB2A* in roots (A) and leaves (B) of control and water stressed *P.sativum* plants. C control; D1- dehydrated for 7 days; D2- dehydrated for 10 days;R-rehydrated plants. Data points represent the average of three different measurements ± standard errors based on three replicates. Significant differences ($p \leq 0.05$) between treatments are denoted with different lower case letters.

(*Dendranthema vestitum*) demonstrated that the expression of *DvDREB2A* changed during dehydration treatment, reaching maximum level after 6 hours, but was still higher after 4 hours and 12 hours (Liu et al., 2008). A very interesting fact is that the level of *DvDREB2A* was lower after 2 hours in comparison with control. But these data should be interpreted very carefully, taking into account the fact that the authors used PEG 6000 solution for induction of dehydration. Some experiments using overexpression of DREB2A transcription factor showed significant changes in stress tolerance, especially regarding drought and osmotic stress (mainly salt stress) (Agarwal et al, 2010; Mizoi et al., 2012; Chen et al., 2007). Plants with overexpressed *DREB2A* became stress tolerant; overexpression of an active form of *GmDREB2A* causes growth defects in the germination and seedlings stages (Mizoi et al., 2012). In addition, transgenic *M. truncatula* MtDREB2A (with a constitutively active form of MtDREB2A) plants exhibit significantly dwarfed seedlings. In addition, according to literature data (Lata and Prasad, 2011) DREB2A is involved in ABA-independent plant stress response. However, our unpublished data on pea plants grown on nutrient medium showed that the treatment with 100 μM ABA induced the expression of DREB2A. All these data suggest the existence of fine regulation between the expression of DREB2A gene and its downstream stress-responsive. Here, we tried to test the response to dehydration in the conditions closely similar to field conditions.

Materials and methods

Plant material

Pea (*Pisum sativum* var.*sativum* cultivar " NS MRAZ") was used throughout this study. Ten seeds were sown in plastic pots and regularly watered with 200 ml of water. Plants were grown in the greenhouse of the Institute of Molecular Genetics and Genetic Engineering in Belgrade under optimal conditions. Plants were sown in October. Fifteen-day old pea plants were subjected to drought stress. Stress treatment was performed by withholding irrigation during 7 and 10 days. After 10 days, the stressed plants were recovered by watering. After each treatment the samples were collected and immediately frozen in liquid nitrogen and stored at -70°C for further analyses. Unstressed "controlled" plants were regularly watered during 10 days, and harvested in parallel to obtain the same tissues at the same time interval. To avoid influence of potential circadian rhythm, all samples were harvested at the same time at day (10^{h}).

Estimation of water content

Relative water content (RWC) in leaves was determined for each control and drought treatment and it was calculated according to Barrs and Weatherly (1962):
RWC (%) = [(fresh weight - dry weight) / (saturated weight - dry weight)] X 100
Leaf dry weight (DW) was measured after oven drying at 105°C for 24 hours, and the saturated weight was measured after incubation of the leaves in moist filter paper for 24 hours in Petri dishes at room temperature.

Lipid peroxidation assay

The level of lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction as described by Heath and Packer (1986). The MDA content was expressed as nmol MDA/DW.

ROS analysis

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a molecular probe for ROS levels in the cell (Allan and Fluhr, 1997; Collen and Davison, 1997). For analysis, we used leaves washed with distilled water for 5–7 min, to avoid artificial surface ROS production. After that, leaves were dried on filter paper and fresh weight was determined. Finally, ichlorofluorescein (DCF) content was expressed according to standard DCF concentration curve, in nmol min^{-1}g DW.

Total RNA isolation and cDNA synthesis
Total RNA from all samples were isolated using GENEJET RNA isolation kit (ThermoScientific, Lithuania), according to manufacturer's protocols from different pools of five plants to minimize the individual plant variation in gene expression. The integrity of isolated RNA was checked on formaldehyde 2% (w/v) agaroze gel. The quality as well as the purity of isolated RNA was determined by measuring optical density at 230 nm, 260 nm and 280 nm, A_{260}/A_{280} and A_{260}/A_{230} ratio, using NanoVue spectrophotometer (GE Heltcare, USA). Only the RNA samples with A_{260}/A_{280} ratio between 1.9 and 2.1, and with A_{260}/A_{230} ratio grater then 2.0 were used in further analyses. To avoid any genomic DNA (gDNA contamination), total RNA was treated with DNAfree (Ambion, USA), according to manufaturer's instructions. Absence of genomic DNA in RNA samples were tested before reverse trannscription by PCR using primers designed to amplify the 111bp intron fragment of phospolipase C gene (*PLC*), according to Die et al. (2010). Total RNA (1µg) was reverse transcribed using RevertAid reverse transcriptase (ThermoScientific, Lithuania), according to the manufacture's protocol, using random hexamers.

Primer design and amplification of DREB2A

PCR primers for amplification of part of *DREB2A* were designed using different available free programs (Primer3, ITD, GeneFisher). We used the *DREB2A* cDNA sequence from model legume *Medicago truncatula* (DQ908959.1). Primers were tested using cDNA from treated plants. Twenty-five microliter RT-PCR was carried out in Biometra Thermocycler PCR maschine (Bimetra, Germany) with High-Fidelity PCR Enzyme Mix (ThermoScientific, Lithuania), using 5U High Fidelity PCR Enzyme Mix, 10X High Fidelity buffer with 15 mM $MgCl_2$, 10 pmol of each primers (D1 f 5'-TAAACCAAAGCGTAAAGCACCGGC-3' and D1r 3'-TCATCAGGCTCATCCATTGGCTCT-5') and 50 ng cDNA. The temperature profile consisted of an initial denaturation of 5 min at 95°C followed by 35 cycles at 95°C for 30 s, 49°C for 30 s (annealing) and 72°C for 2 min (extension/elongation), followed by a final step of 10 min at 72°C. PCR products were separated on 2% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light. As a control, we used genomic DNA from *M.truncatula*. Only succesfully amplified PCR products were then purified using QIAQUICK PCR purification kit (Qiagen,USA), and sequenced using GeneticAnalyser 3300. The obtained sequence was alligned using BLAST, and submitted to gene bank.

Quntitative real time PCR

The Real time quantitative RT-PCR amplification of *DREB2A* gene was performed using the first strand cDNA, synthesized from RNA samples collected from control (unstressed) and plants exposed to different level of dehydration, as well as to recovery. Polymerase chain reactions were performed in a 96-well plate with ABI PRISM 7500 (Applied Biosystem, USA), using TaqMan MGB-probe based technology. As a reference gene, we used β-tubulin (Die et al., 2010). Primers and probes for *DREB2A* and reference gene were designed using the Assay-by-Design (Applied Biosystem, USA). For each PCR reaction a final volume of 20 µL was used, containing 1µL of cDNA (50 ng coming from RNA reverse transcribed), 10 µL of 2x taqMan PCR MasterMix (Applied Biosystem, USA) and 1 µL of 20x TaqMan Gene Expression Assay (Applied Biosystem, USA). Universal thermal cycling conditions were used. At the end of PCR cycles, the products were analysed through a meltcurve analysis to check the specificity of PCR amplification.Three replicates of each reaction were performed, and data were analyzed according to Livak et al. (2001) and expressed as a normalized expression ratio ($2^{-\Delta\Delta Ct}$) of *DREB2A* gene to specific stress treatment.

Statistical analysis

The data on ten plants of control (unstressed) and dehydrated, as well as recovered plants were collected. All investigated parameters were determined in three independent experiments using three replications. One way ANOVA and Tukey's multiple comparison tests were used to test differences between the means. All calculations were performed using statistical software Sigma Stat. Comparisons with $p \leq 0.05$ were considered significantly different. In all the figures, the spread of values is shown as error bars representing standard errors of the means.

Conclusion

The expression analysis of *PsDREB2A* shows that the gene is strongly induced by drought in root tissues, especially during first dehydration period. Thus, PsDREB2A could be an important transcription factor that can be used for improving abiotic stress tolerance and for estimating the difference between genotypes.

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