

**SZENT ISTVÁN UNIVERSITY**

**INVESTIGATION OF DROUGHT TOLERANCE IN DIFFERENT  
WHEAT VARIETIES**

Doctoral (Ph.D) Thesis

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**Name of Doctoral School:** Doctoral School of Horticultural Science

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## 1. BACKGROUND AND AIMS

Common wheat (*Triticum aestivum* L.) is highly vulnerable to drought stress, especially during the reproductive phase. Drought tolerance is multifactorial trait, which depends on interactions between genotype and environmental factors.

A current aim of plant research is to discover key factors of survival in dry conditions that are common in regions with limited irrigation capacities or those affected by frequent drought. Scientists therefore strive to understand the underlying mechanisms of drought tolerance in plants. Plant breeding is aimed at increasing yield under extreme environmental challenges especially in drought-affected regions.

In order to understand the molecular mechanisms of tolerant response of plants in drought, investigations need to be conducted at the molecular level. Drought acts primarily on growth and subsequently on photosynthesis. It is known that the yield/amount of grains is largely dependent on the latter. It is widely accepted that yield is affected by the degree and location of the remaining photosynthesis, especially in case of flag leaves.

Increased wax deposition in the cuticle has also been frequently observed upon drought-stress. Plants with higher tolerance towards water stress often possess thicker and less water permeable cuticular layer than those being more sensitive (Shepherd and Griffiths, 2006).

Upon stress alterations in signalling networks drive metabolic and physiological changes in the affected plants. Thus, gene-expression patterns change, for example dehydrin genes often became upregulated during drought. These genes' products help to evade the harmful effects of water loss by stabilizing membranes upon stress (Graether and Boddington, 2014). Dehydrin proteins may also prevent the damage caused by reactive oxygen species in case of water-deprivation (Rorat, 2006).

Transcription factors (TF) are key regulatory elements of gene-expression. Multiple TFs often cooperate on stress-induced genes suggesting that different transcriptional regulatory mechanisms are involved in signalling pathways of drought, cold or salt stress (Kauzo et al, 2003).

A well established, characteristic response to water-deprivation of plants is biosynthesis of the hormone abscisic acid (ABA). The main role of ABA is to regulate the

water-relations of plants and to improve the tolerance towards osmotic stress. ABA induces stomata to close upon water shortage in order to prevent intracellular loss of water (Mittler et al, 2010).

Our aim was to investigate some physiological and molecular markers associated with tolerance and sensitivity towards water deprivation in two tolerant (Plainsman V. and Mv Emese) and two sensitive (GK Élet and Cappelle Desprez) cultivars of wheat.

In order to find relevant markers that differentiate among the genotypes with contrasting behaviour the following examinations were performed:

- assessment of the synthesised osmo-protectants upon osmotic stress
- measurement of the effect of water-deprivation on gas exchange parameters
- investigation of the expression of drought stress associated genes
- identification of transcriptional factors that may regulate the investigated stress related genes
- establishing sensitivity of the cultivars towards abscisic acid that may play a key role in protection against desiccation.
- Furthermore, we aimed to identify and clone a gene of wheat that may be responsible for the establishment of the cuticular wax-coverage which is crucial in drought-tolerance. We wanted to define the expression pattern of this gene in different organs of mature wheat plant, as well as in the flag leaves during the course of a water-deprivation period.

## **2. MATERIALS AND METHODS**

### **Plant material**

For water stress treatment plants of the winter wheat varieties under investigation were planted in a soil-sand-peat mixture (3 : 1 : 1, v/v/v). After 7 weeks of vernalisation at a temperature of 2°C plants were grown in PGV-15 growth chambers (Convion, Winnipeg, Canada) using the spring climatic program T1 (Jäger et al. 2015). Drought stress was generated by total water withholding. The control plants were grown on 21°C, under 10/14 h light/dark cycle.

For the osmotic stress treatment with polyethylene glycol seeds were grown in Petri dishes in a light room. After ten days seedlings with well developed shoot and root were put in a hydroponic system into Hoagland solution at pH: 5,8 (Hoagland és Arnon, 1950).

The control group and treated seedlings developed in Hoagland solution for the first 10 days. Thereafter for stress treated plants Hoagland solution was supplemented with PEG and its concentration was increased weekly. PEG concentration was gradually increased from 8 w/v % to 12 w/v %, subsequently to 15 w/v % and finally to 18 w/v%.

For *in vitro* ABA treatment the plants were treated according to the method of Kurahashi et al. 2009. Germinated seedlings were transferred into Petri dishes with filter paper wetted with distilled water or with 10, 20 or 50  $\mu$ M ABA solution, and were grown further on a steady 23°C temperature and under 12/12 h light/dark cycle.

### **Measurement of photosynthesis parameters on drought treated wheat plants**

Investigation of gas exchange was performed by an LCI instrument every other day during the water stress period. Flag leaves of plants were measured because they keep metabolic activity for longer than other leaves.

### **Determination of relative water content (RWC) of leaves**

The effect of the highest osmotic stress applied (18% PEG treatment) on the leaves was determined by measuring relative water content (RWC) of leaf tissues. Samples were taken from the middle of flag leaves.

### **Statistical analysis**

For evaluation of results statistical tests were used in order to discriminate significant differences. In the abscisic acid treatment experiment Welch's ANOVA test was used. RWC results were analysed by the T-test of Microsoft Office 2013 Excel program. One-way ANOVA with post-hoc Tukey HSD test was used to establish significant differences among the expression levels of genes in qRT-PCR.

### **RNA isolation, equalization of RNA amounts and cDNA synthesis**

RNA extraction of drought stressed as well as non-stressed plants was done with TRI Reagent (Molecular Research Center, Inc.). The concentrations of RNA samples were equalized according to measurements with a NanoDrop equipment. First strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) was used for reverse transcription of the RNA samples.

## **RT-PCR and real-time PCR (qPCR)**

Before RT-PCR experiments with target genes, the concentration of cDNAs were equalized by using control primers. PCR products were separated on 1,5% agarose gels. Real time PCR was performed on a Rotor Gene 6000 instrument.

## **Checking of genomic DNA contamination of cDNA samples**

DNA contamination of cDNA samples were examined by using intron spanning primers. This method made sure that only those genes were analysed which were expressed at the moment and produced mature mRNA.

## **Selection and cloning of the wheat sequence homologous to the *Arabidopsis* WIN/SHN1 (*WAX INDUCER/SHINE1*) gene**

One of the aims of our researcher team was to identify and characterise a potential WIN/SHN type regulator of cuticle formation in wheat (Jäger *et al.* 2015).

Similar sequences to the *Arabidopsis* WIN/SHN1 gene (NP\_172988.1) were looked for in the *Triticum aestivum* subset of the NCBI EST database by BLAST search. Based on these EST sequences a particular entry in the triDFLB database (tplb0011g14) was identified. Appropriate primers were designed by the Primer Premier program in order to amplify the full length ORF of this wheat WIN/SHN1 orthologue candidate gene.

The wheat gene was found expressed in *in vitro* grown plantlets, *TaSHN1* ORF was amplified by RT-PCR and cut by appropriate restriction enzymes. The cut fragment was ligated into pBluescript KSII vector. The next step was transformation of the construct into *Escherichia coli* bacteria. Selection for transformed and recombinant bacteria was done by Ampicilin (100µg/ml) and IPTG/X-Gal respectively. Bacterial strains containing the vector with cloned *TaSHN1* cDNA fragments were obtained as result.

## **Analysis of gene expression data**

For evaluation of qPCR results  $C_T$  (Threshold cycle =  $C_T$ ) values were used. Determination of differences among the samples was possible based on these values. Results were quantified according to this equation:

$$\Delta C_T = C_T(\text{target gene}) - C_T(\text{reference})$$

where the  $\Delta C_T$  value is the difference between the threshold cycle number of control and treated samples. After establishing  $\Delta C_T$  value the expression differences can be calculated by the following equation:

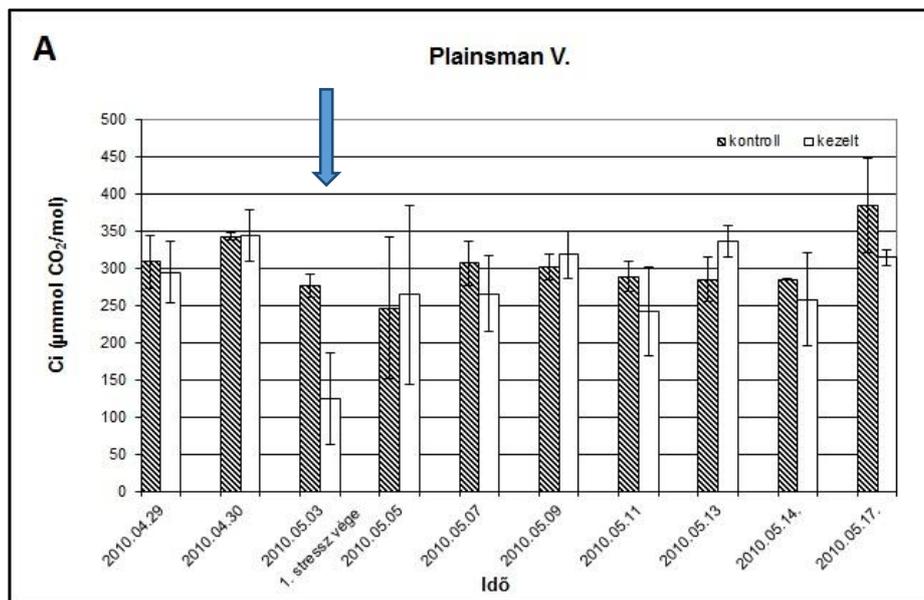
$$2^{-\Delta C_T}$$

This value represents a ratio between the expression level of the examined genes compared to the reference gene (TaL in our case).

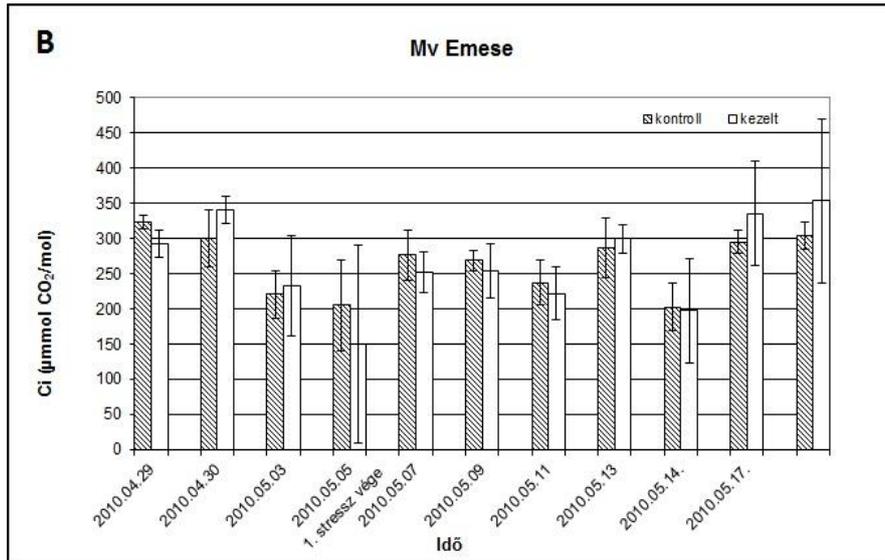
### 3. RESULTS

#### Measuring gas exchange parameters of drought treated wheat

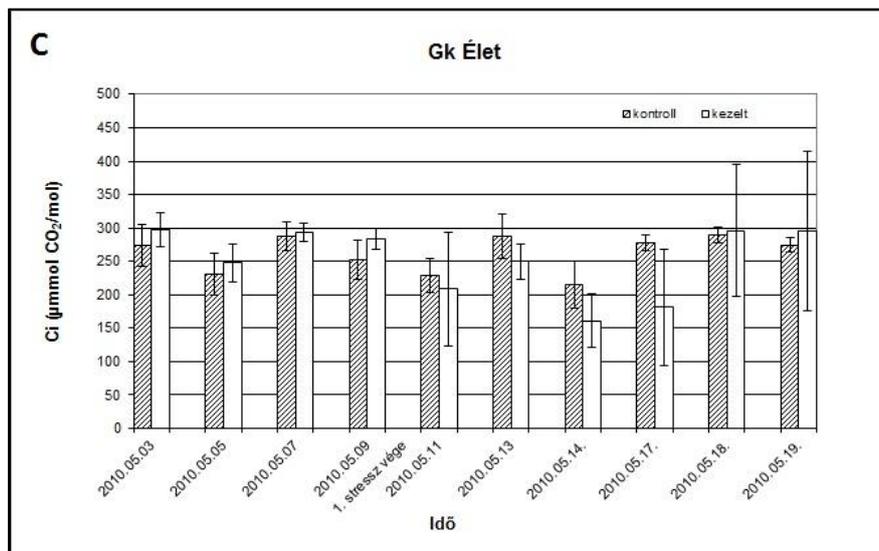
The photosynthetic apparatus is relatively protected against water stress, nevertheless it can be damaged in case of serious or long stress. Measuring gas exchange parameters gave us a possibility to determine water status as well as the rate of damage done on the photosynthetic apparatus. In **Fig. 1, 2, 3, 4**  $C_i$  values of stress treated and control plants are presented during two cycles of water withholding at anthesis. Values in the figures show averages  $\pm$  SD.



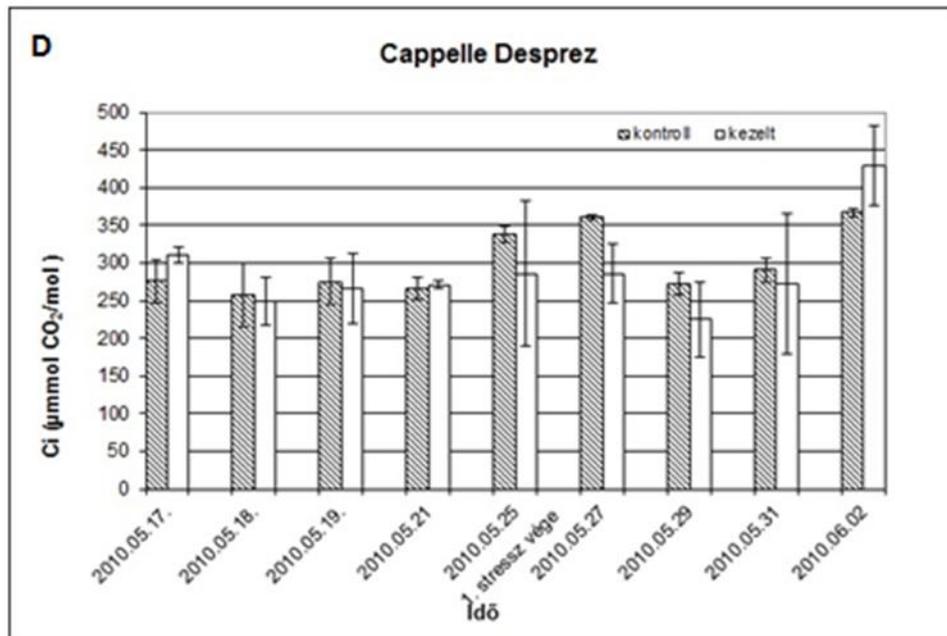
**Figure 1:** Intercellular CO<sub>2</sub> concentration ( $C_i$ ) of Plainsman V.



**Figure 2:** Intercellular CO<sub>2</sub> concentration (Ci) of Mv Emese



**Figure 3:** Intercellular CO<sub>2</sub> concentration (Ci) of Gk Élet



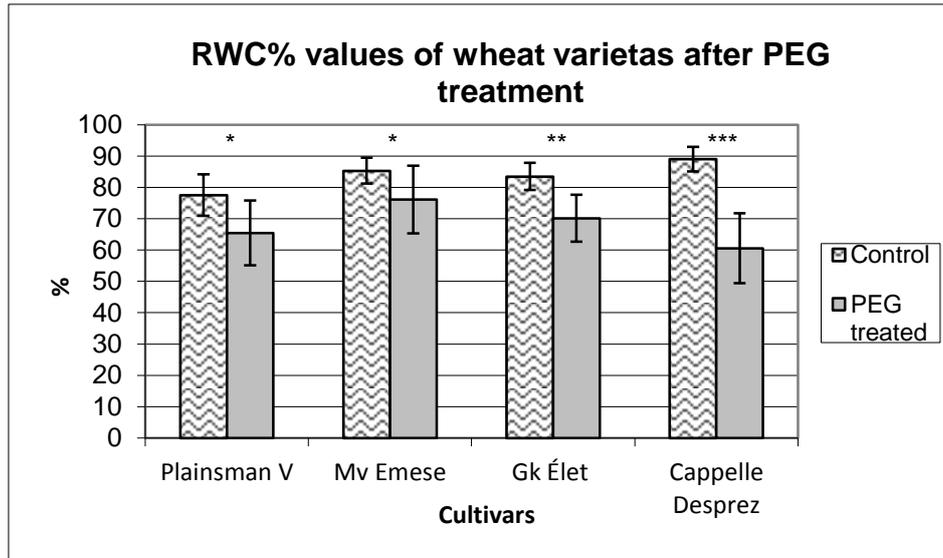
**Figure 4:** Intercellular CO<sub>2</sub> concentration (Ci) of Cappelle Desprez

Based on the data a decline of the Ci value of treated plants in the tolerant genotype Plainsman could be observed at the end of the first stress treatment (blue arrow in Figure 1). This decline of Ci did not occur in the sensitive cultivars (cvs. Gk Élet, Cappelle Desprez) or in Mv Emese.

The contrasting values of Ci at the end of the first drought period can be explained by faster stomatal closure and sustained photosynthetic activity in the drought tolerant cultivar Plainsman, which depletes the leaf interior of CO<sub>2</sub>.

#### **Treatment of wheat seedlings with PEG**

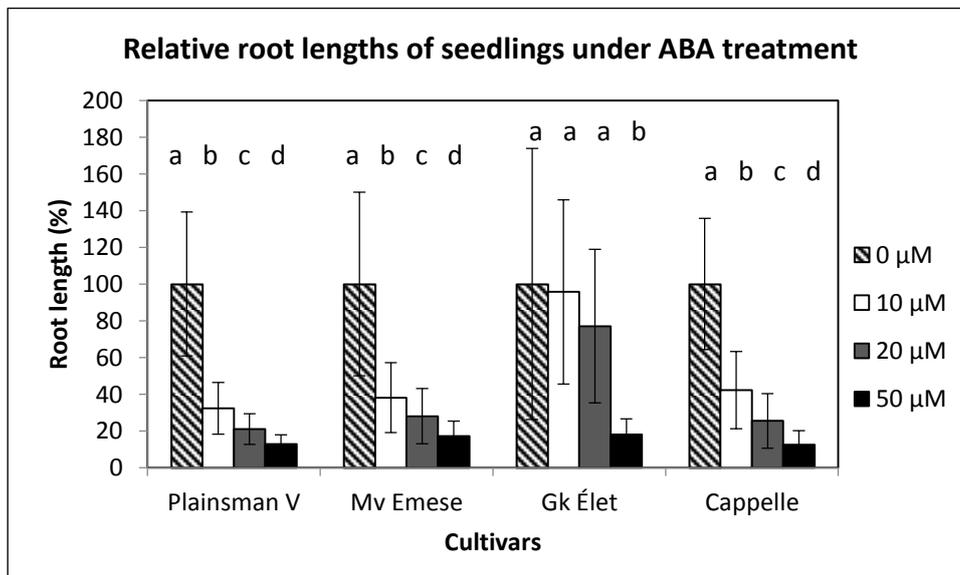
Contrary to our expectations the Plainsman V variety did not exhibit high RWC values among the genotypes after 18% PEG treatment (**Fig. 5**). In case of the sensitive varieties, Gk Élet showed higher RWC% levels in comparison to that of Cappelle Desprez.



**Figure 5.** Relative water content (RWC) of leaves at the end of the treatment. Means  $\pm$  standard deviations are shown. Significant level: \*\*\*  $p \leq 0,001$ ; \*\* $p \leq 0,01$ , \* $p \leq 0,05$ .

### Examining ABA sensitivity of seedlings

Measuring inhibition of root length growth at the seedling stage in response to external ABA is a simple method for characterization of ABA sensitivity (Thole *et al.* 2014). We used this approach on the selected wheat cultivars (**Fig 6.**).

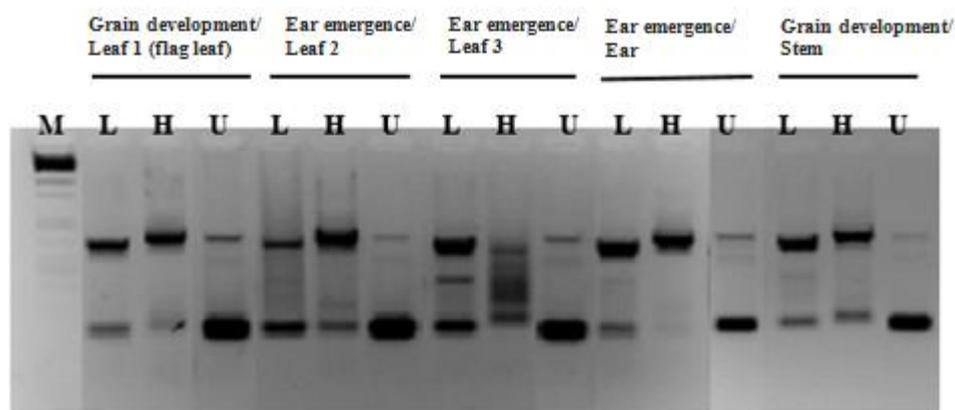


**Figure 6.** Relative root lengths of seedlings after one week growth at the indicated ABA concentrations (10, 20 and 50  $\mu\text{M}$ ) were compared to untreated controls.

Exogenous application of ABA at low concentrations retarded root elongation to a different degree. Cultivar GK Élet showed significantly the weakest response to 10 and 20  $\mu\text{M}$  ABA applications. Different letters indicate differences at  $p < 0.05$  of probability

### Testing the applicability of control RT-PCR primers in wheat

Trustworthy gene expression data require the use of appropriate constitutive genes. Such genes as well as PCR primers were suggested by Paolacci *et al.* (2009), some of those were tested in several wheat samples we used (**Fig. 7**).



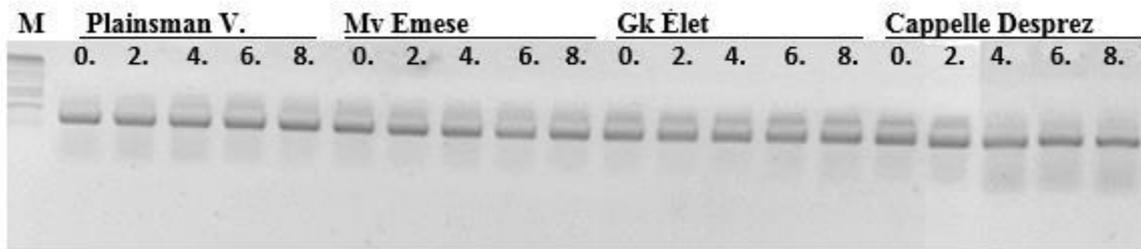
**Figure 7.** RT-PCR results with three potential control primer pairs on cDNA samples from different wheat organs. Primer pairs: L – TaL; H – TaH; U – ubiquitin, Molecular weight marker: M

Results showed that the TaH (\*H) gene sequence could be amplified with the suggested primer pair reliably under the condition used. This gene's ubiquitous expression could be ascertained in our experiment, therefore it was appropriate choice for our purposes.

### Checking genomic DNA contamination in cDNA samples

This quality control step in the experiments requires a transcribed target gene sequence with an intron, which is removed during mRNA splicing. Genomic DNA (gDNA) and cDNA give higher (intron containing) and lower molecular weight (lacking intron) PCR products respectively. For wheat we used an intron spanning primer pair (TaG), as suggested by Ciaffi and co-workers (2006). In this case PCR product from gDNA could be expected 618 bp long while a 266 bp fragment was produced from cDNA. Results showed that the PCR template

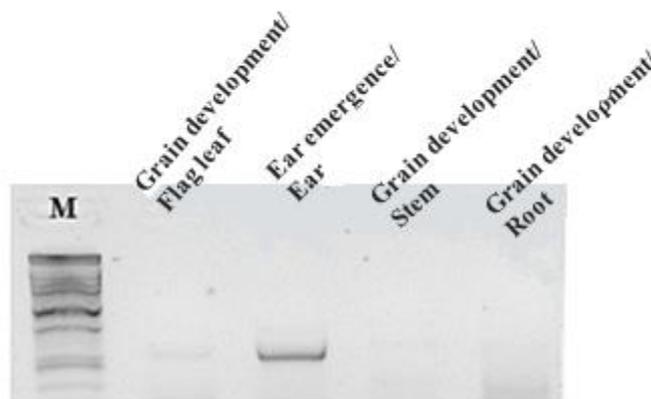
didn't contain intron sequence, therefore our samples were free of gDNA contamination (**Fig. 8**).



**Figure 8.** RT-PCR amplification products on water stress treated wheat samples using TaG intron spanning primer pair. Molecular Weight Marker: M

### Investigation of *TaSHN1* gene expression in different organs of wheat

Based on sequence homology we selected *WIN/SHINE1* like ESTs from the wheat subset of the NCBI database with the help of the BLAST program. Based on the EST sequences the tpb0011g14 gene was selected from the triDFLB database. Then primers were used to amplify the full length ORF of this gene and this DNA fragment was cloned. Our further aim was to determine the organ specific expression pattern of this gene (**Fig. 9**). It is known that the *WIN/SHN1* gene is expressed at high intensity in the flower of *Arabidopsis*.



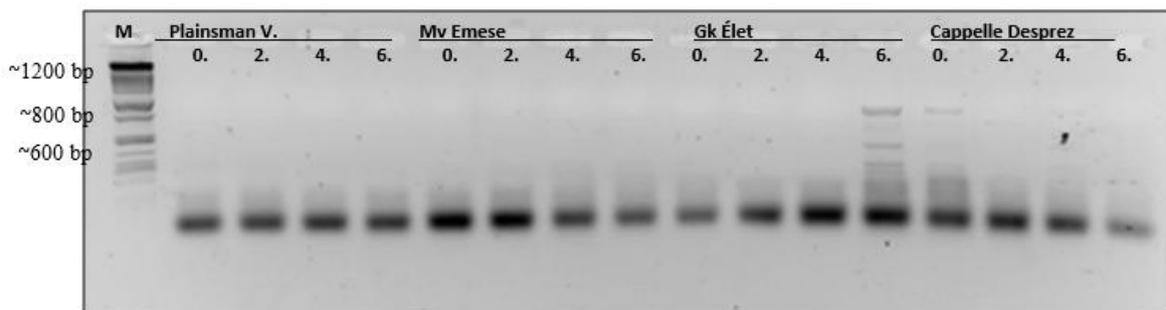
**Figure 9.** Expression pattern of the *TaSHN1* gene in different developmental phases and in different organs of wheat. Marker (M).

Transcription of *TaSHN1* which is homologous to the *Arabidopsis WIN/SHN1* gene was found in the ear at head emergence developmental state. Consequently, our hypothesis seemed to be supported because the homologous *Arabidopsis* gene was also expressed mainly

in flower, suggesting an orthologous relationship. This strengthens the view that the two genes may fulfill similar functions in wheat and *Arabidopsis*.

### Testing *TaSHN1* gene expression level in water stressed wheat cultivars

The *Arabidopsis* *WIN/SHN1* gene participates in cuticle formation, which is accelerated by water stress in the wheat cultivars studied. Therefore, a wheat orthologue of this gene was supposed to be induced by water deprivation in case it is involved in the tolerance response. To test this hypothesis expression of *TaSHN1* was tested in water stressed wheat leaves of the four varieties studied.



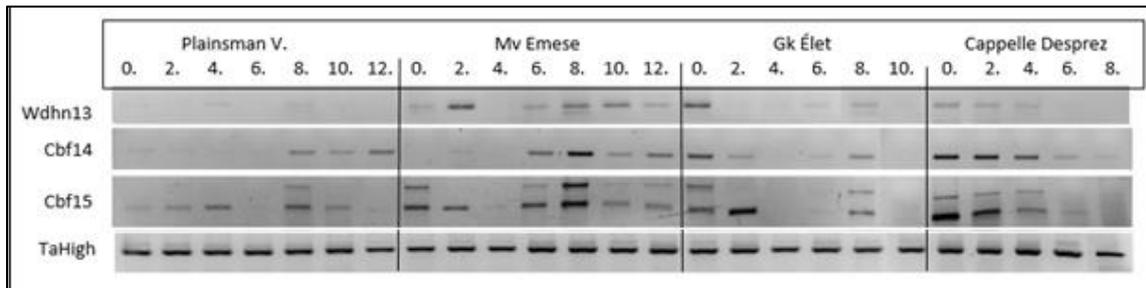
**Figure 10.** RT-PCR reaction with *TaSHN1* gene specific primer pair (buSWk1 and buSWk2). Numbers indicate days of water stress treatment.

We expected a 618 bp long PCR fragment to appear, but no product around this size was amplified in most samples (**Fig. 10**). It means that the *TaSHN1* gene was not induced in response to water stress. We hypothesize that other potential regulators may be responsible for the increased production of wax on flag leaf surfaces.

### Investigation of transcription factors and defence gene expression during water stress

We examined genes with well-known roles in water stress response. We were curious how expression level of some selected genes change in different varieties and if there is any connection between expression of dehydrin genes and CBF transcription factors (**Fig. 11**).

We tested C-repeat binding factor transcription factors (CBF's) because expression of these genes was related to dehydrin gene induction for example in *Brachypodium distachyon* drought tolerance response (Ryu *et al.* 2014).

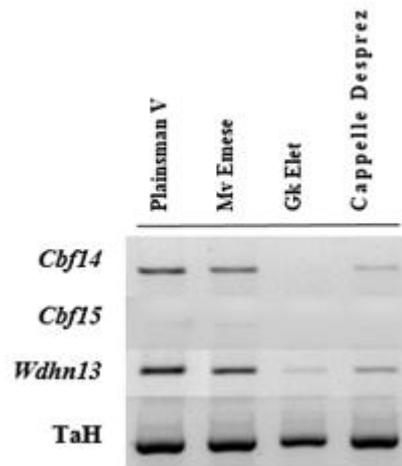


**Figure 11.** RT-PCR products of the studied genes in the four genotypes during drought stress treatment. Numbers indicate days of treatment.

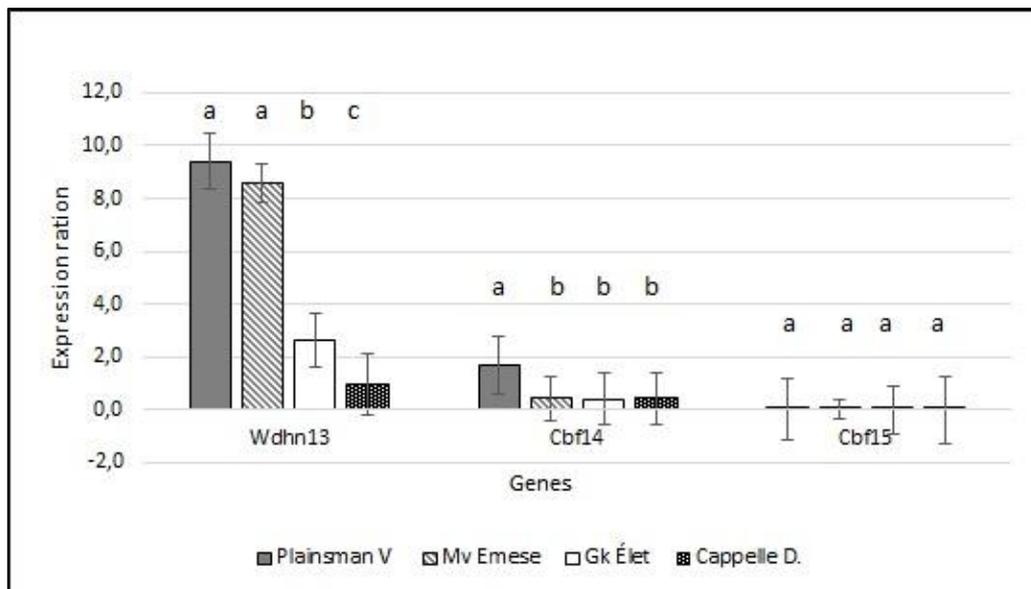
The most striking result of semi quantitative RT-PCRs of all studied genes was a late wave of induction around the 8th day of treatment, except for the most sensitive Cappelle Desprez, where this induction did not occur. Gene specific RT-PCR product from the dehydrin *Wdhn13* was induced in Mv Emese at 6-10th days of water deprivation. mRNA of this gene was barely detectable in the other three genotypes, with low level of induction in Plainsman V and Gk Élet. Parallel changes in expression of *Cbf14*, *Cbf15* and *Wdhn13* genes were noted, but this effect was not stringent. In order to confirm identity of the PCR products representative RT-PCR fragments of *Cbf14*, *Cbf15* and *Wdhn13* were extracted from agarose gels and sequenced directly.

#### **Transcript abundance of *Cbf14*, *Cbf15* and *Wdhn13* genes in non-stressed seedlings**

Expression pattern of the genes was investigated in leaves of 3 week old non-stressed seedlings (**Fig. 12**). According to RT-PCR transcription rate of *Wdhn13* and *CBF14* genes correlated among the cultivars. mRNA of the *Cbf15* gene however was not detected at all under these conditions. Quantitative RT-PCR was also performed on cDNA samples prepared from unstressed seedlings, which had been also used in RT-PCR. Data largely confirm results of RT-PCR regarding cultivar specific differences. High level expression of *Wdhn13* was found exclusively in the tolerant cultivars (Plainsman V and Emese) (**Fig. 13**). mRNA levels of the *CBF14* gene were generally modest, highest in the cultivar Plainsman V. mRNA of the *Cbf15* gene however was not detected at all under these conditions, or its level was extremely low.



**Figure 12.** RT-PCR products for the studied genes in leaves of non-stressed seedlings of the four genotypes.



**Figure 13.** Expression levels of the studied genes in unstressed seedlings as measured by qRT-PCR. Different letters indicate differences in expression intensity among cultivars at  $p < 0.05$  of probability for each gene separately

### 3.1. New Scientific Results

- CO<sub>2</sub> concentration in the flag leaves of the Plainsman V cultivar declined sharply at a late stage of drought stress discriminating this genotype from all other cultivar studied

- Reliable control genes for RT-PCR and qPCR experiments were identified in wheat, moreover primers were used that enabled us to screen genomic DNA contamination in cDNA samples
- *Wdhn13* dehydrin as well as *Cbf14* and *Cbf15* transcription factor genes were found co-ordinately upregulated upon water-deprivation at a late stage of drought treatment. This effect was the highest in the Mv Emese tolerant cultivar.
- In non-stressed seedlings expression of the *Wdhn13* gene was highest in leaves of the tolerant genotypes (Plainsman V, Mv Emese). Expression of the *Cbf15* gene was not detected in unstressed seedlings of any cultivars studied
- A wheat orthologue gene of the known *Arabidopsis* transcriptional factor *WIN/SHN1*, responsible for cuticle development, was identified *in silico*. The gene was cloned and its expressional pattern in different wheat organs was described. Furthermore, we showed that the gene was not expressed in the middle part of flag leaves in water stressed wheat plants
- Cultivar Gk Élet was shown relatively insensitive to abscisic acid induced root growth retardation in the seedling stage in comparison to the other genotypes tested

#### 4. CONCLUSIONS AND RECOMMENDATIONS

##### **Effect of water deprivation on gas exchange of the wheat genotypes**

Among the investigated cultivars the  $C_i$  value decreased more sharply in case of Plainsman V after the first water-deprivation period. This result was confirmed by Fábíán et al (2013) and Paul et al (2016). In their experimental setup the same cultivars were investigated by withholding water. Thus, results of other researchers on the properties of Plainsman V are in agreement with our data and support our conclusions. In case of the drought-sensitive Cappelle Desprez genotype however we could not detect a decrease of the  $C_i$  value, in contrast to the observations of Fabian et al (2013). In conclusion we hypothesize that among the investigated genotypes the drought-resistant Plainsman V is able to maintain photosynthesis longer at the end of the water-deprivation period. Due to sustained high level of  $CO_2$  fixation and stomatal closure low  $C_i$  values are detectable in this genotype.

##### **Investigation of the effect of osmotic stress**

Guóth et al (2010) investigated the response of drought-tolerant Mv Emese and drought-sensitive Gk Élet genotypes to osmotic stress, caused by 400 mOsm polyethylene glycol. The water-potential in Mv Emese has not changed significantly in the stressful environment during the 21 day-long experiment. However, in case of Gk Élet the water-potential significantly decreased under the same conditions. Furthermore, the water potential values of the leaves were lower compared to the controls in both cases. Our results were in accordance with the above-mentioned observations such as the Gk Élet is sensitive, thus its response to osmotic stress is higher than the drought-tolerant Mv Emese genotype. Although the Plainsman V is known as drought-tolerant species, it could not tolerate osmotic stress well, presumably the underlying method of its tolerance is different.

### **Root growth response of seedlings in solutions with different concentration of abscisic acid**

More intense root growth of Gk Élet seedlings were observed in 10 and 20  $\mu\text{M}$  external ABA hormone concentrations, compared to the other genotypes. The observed insensitivity to ABA hormonal responses may contribute to the lower drought-tolerance level of this cultivar. This insensitivity to ABA induced root-growth retardation of Gk Élet cultivar was also observed by Guóth et al (2010). Via externally applied PEG treatment they noticed that the relatively high level of endogenous ABA inhibited less the root-growth in Gk Élet, than in the tolerant Emese genotype.

### **The investigation of WIN/SHN1 gene in different stages of wheat development**

It is known that the *WAX INDUCER/SHINE (WIN/SHN)* gene plays a key role in the development of cuticle of *Arabidopsis*. This regulator is an APETALA2/ERF-type transcription factor (Aharoni *et al.* 2004). Broun et al (2004) investigated the expression of this gene in *Arabidopsis*. They found its highest level of expression in the flower by RT-PCR, while the expression was low in the leaves and in the stalk.

The expression of the wheat *WIN/SHN1* homolog *TaSHN1* gene was also highest in the flower of the plants. This corroborated our assumption that the two genes are true orthologues. High level of *WIN/SHN1* gene expression in flowers is not surprising, for during the fast development of flower parts cuticle components and waxes should also form faster, requiring high activity level of related genes.

### **Expression of *TaSHN1* in drought-exposed wheat**

We applied drought-stress on four wheat varieties in order to investigate the possible relationship between stress associated increase of wax-coverage and induction of the *TaSHN1* gene. We performed RT-PCR on leaf samples both from stress-exposed and control plants. The RT-PCR reaction was specific to the *TaSHN1* gene, a wheat member of the *WIN/SHN1* transcription factor family. We did not observe any indication of increased *TaSHN1* gene expression during water stress in mid leaf regions of the varieties tested. Presumably in wheat other regulatory genes and transcriptional factors play more important role in regulation of accelerated cuticle formation upon drought-stress.

### **Analysis of drought-induced gene expression**

Apart from physiological measurements, we also assessed changes in expression of some supposedly stress associated genes in the investigated wheat varieties. Drought tolerance level of the four varieties studied have been well established. Water stress tolerance of Plainsman V and Mv Emese and sensitivity of Gk Élet and Cappelle Desprez were described by Guóth *et al.* (2009) and Jäger *et al.* (2014). Results show that upon water-deprivation the expression of *Wdhn13*, *Cbf14* and *Cbf15* genes are increased in all varieties except in Cappel Desprez. This expression seems to take place in a late phase of stress treatment, genes are upregulated approximately a week after the onset of total water-deprivation. This late wave of gene expression was most pronounced in the tolerant Mv Emese variety.

An association between expression of *DHN* and *Cbf* genes has already been described, eg. upon cold-stress (Kume *et al.*, 2005). Largely coordinated expression of *Wdhn13* and *Cbf* genes during water stress points to the possibility of a regulatory role of *Cbf* genes (especially *Cbf14*) over *Wdhn13*.

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## **PUBLICATIONS IN CONNECTION WITH THE DISSERTATION**

### **Impact factory reviews**

Cs. Deák, K. Jäger, V. A. Nagy, R. Oszlányi, B. Barnabás, I. Papp (2016) C-repeat binding factor and dehydrin genes are induced co-ordinately in drought tolerance response of wheat cultivars (accepted in *Journal of Agricultural Science*)

### **Publications in peer reviewed journals**

Cs. Deák, K. Jäger, A. Fábrián, V. Nagy, Zs. Albert, A. Miskó, B. Barnabás, I. Papp (2011) Investigation of physiological responses and leaf morphological traits of wheat genotypes with contrasting drought stress tolerance. *Acta Biologica Szegediensis*, 55(1):69-71

Cs. Deák, Katalin Jäger, Attila Fábrián, István Papp (2010) Low and high ways from post-transcriptional RNA regulation to drought tolerance. *Plant Signaling & Behavior* 5(12): 1539 - 1542

### **Additional impact factory reviews**

K. Jäger, A. Fábrián, G. Eitel, L. Szabó, Cs. Deák, B. Barnabás, I. Papp (2014): A morpho-physiological approach differentiates bread wheat cultivars of contrasting tolerance under cyclic water stress. *Journal of Plant Physiology*, 171(14):1256-1266. p.

K. Jäger, A. Miskó, A. Fábrián, Cs. Deák, A. Molnár, D. Polgári, B. Barnabás, I. Papp (2014): Expression of a WIN/SHN type regulator of wheat triggers disorganized proliferation of the Arabidopsis leaf cuticle. *Biologia Plantarum*, 59(1):29-36.

### **Abstracts**

Cs. Deák, K. Jäger, V. Nagy, A. Fábrián, A. Miskó, Zs Albert, B. Barnabás, I. Papp (2012): Morphological traits and physiological responses of four wheat genotypes with contrasting drought susceptibility. Pannonian Plant Biotechnology Workshops Advances in Plant Breeding and Plant. Biotechnology in Central Europe, Jun 4–6, 2012, Debrecen, Hungary, Book of Abstracts, pp 31-32