Molecular biological analysis of the effect of TaCBF14 and TaCBF15 transcription factors in transgenic barley and wheat lines

OTKA PD116564 - Final report

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PURPOSE OF THE PROJECT

The TaCBF14 and TaCBF15 transgenic barley germplasm overexpressing winter wheat CBF genes were developed in our laboratory (during the period of the earlier project 'OTKA75528' of our work group) and were selected after repeated frost tests. The transgenic lines were considered as valuable materials for further molecular biological researches and functional genomics studies. The aim of the planned work was to understand the mechanism of this improved freezing tolerance at the molecular level. The purpose of this project was to identify which genes are under the control of the given two CBF transcription factors by monitoring of their transcriptome profiles, and to analyse which genes belong to the CBF regulon. In detail, we wished to compare the functional differences/parallelisms in the CBF14 and CBF15 regulon. Transcriptome analysis by the RNA Sequencing method determines the target genes and their expression level in the wheat CBF-overexpressing transgenic barley lines comparing with the spring genotype Golden Promise (wild type).

The function of the identified target genes are validated by quantitative Real-Time RT-PCR (Reverse transcription polymerase chain reaction) method. Besides this, an extensive hormonal examination clarified the possible linkage between CBF transcription factors and hormonal regulations. The verification of these results required further physiological analyses. By the end of the project, we wished to deposit the raw data of the RNA Sequencing experiments into a database to be available for the scientist.

WORK PERFORMED

RNA work: RNA Sequencing experiments and gene expression studies

The wild type spring barley (*Hordeum vulgare* L. cultivar Golden Promise) and the most frost tolerant TaCBF14 and TaCBF15 transgenic barley lines were grown in plant growth chamber in the Phytotron of ELKH ATK Agricultural Institute, Martonvásár, Hungary. After 3 weeks control conditions (20/17°C day/night temperature, 12 h illumination, 400 μmol $m^{-2}s^{-1}$ light intensity, 65% relative humidity), plants were treated by cold (4/4°C with the same light and humidity parameters). Samples were collected during the experiment, from control condition, and from short- and long-term cold treatment (after 1h, 24h, 72h, 1 week, 2 weeks, 3 weeks at 4°C) for the transcriptome analysis, and for additional gene expression analyses and hormonal studies. One sample was "pooled" and homogenized from five plants, and three biological replications were applied.

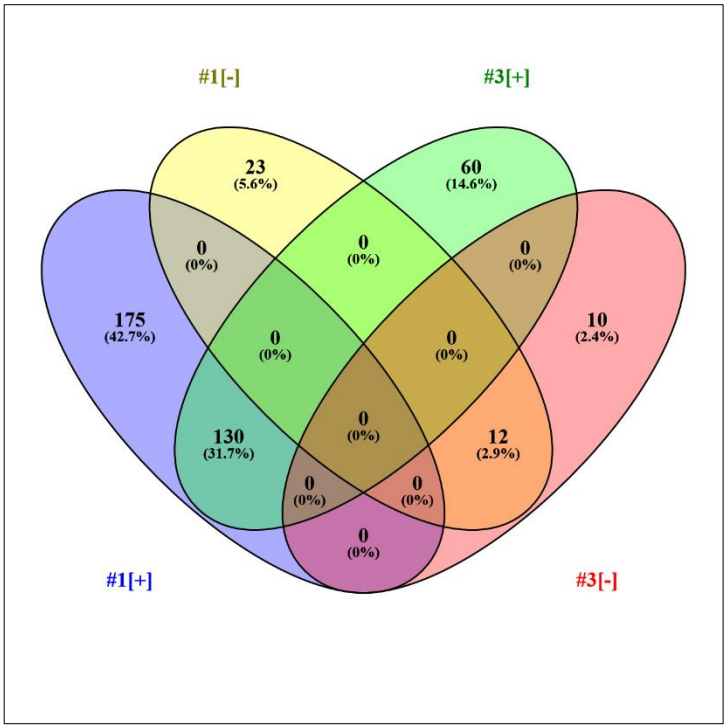
Probe RNA samples were isolated by different methods, using TRIzol® Reagent (Thermo Fisher Scientific); TRI Reagent® combined with Direct-zol™ RNA MiniPrep (Zymo Research) and RNeasy® Plant Mini Kit (Qiagen) to test which method is the best to reach the adequate quality and quantity of RNA for transcriptome analysis. Checking the products, the TRI Reagent® combined with Direct-zol™ RNA MiniPrep kit has been chosen for the isolation of RNA samples. 18 samples (three genotypes, control and short-term cold-treated, in three replications) were prepared for the transcriptome analyses. The quality and the quantity of the

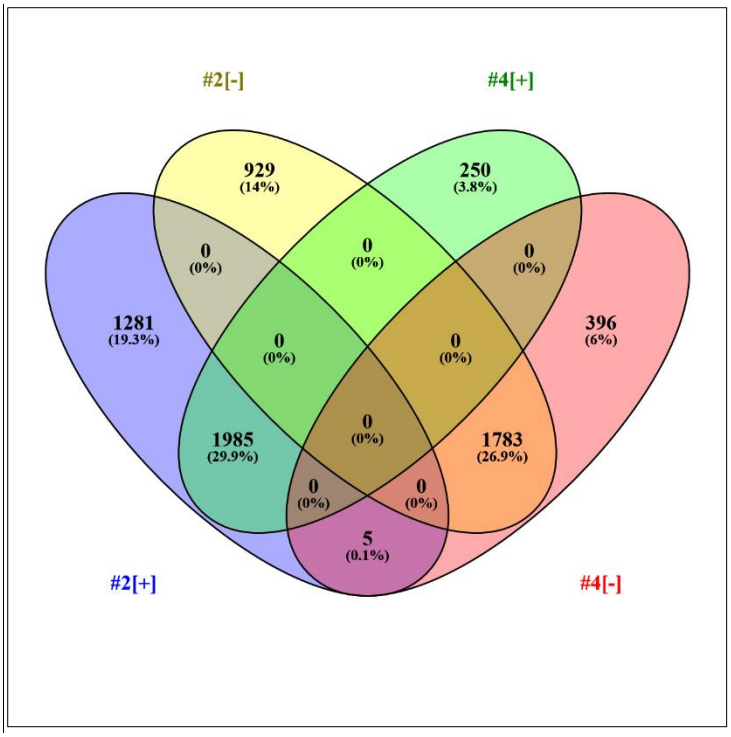
RNA samples has been checked by NanoDrop 2000 Spectrophotometer (Thermo Scientific). The RNA samples (50-50 μg) were precipitated in ice cold 100% ethanol containing 0.1 volume of 3M sodium acetate and sent to the research group of Prof Tamás Dalmay (Head of School, Professor of RNA Biology, School of Biological Sciences, University of East Anglia, Norwich, United Kingdom), who has prepared them for the RNA Sequencing analysis. For mRNA library construction Illumina TruSeq RNA library kit was applied according to the manufacturer's protocol. The RNA Sequencing analysis was performed in Earlham Institute, Norwich, UK. Pooled libraries were diluted to 2 pM for 150bp paired-end sequencing with 150-cycle on two lanes of Illumina 150PE HiSeq4000 (Illumina, San Diego, CA, USA).

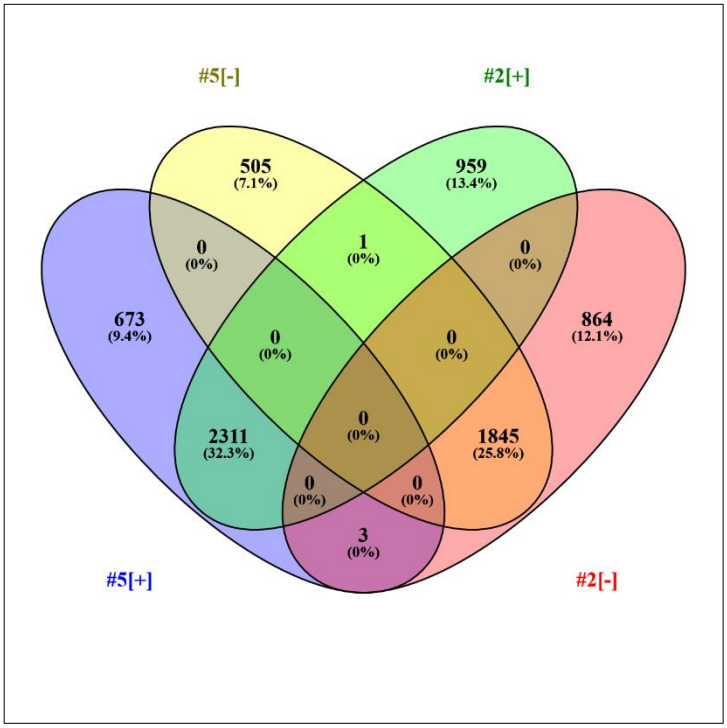
18 RNA samples were sequenced, and 117 GB sequence data were reached. The high throughput sequence alignment was already executed with the help of my colleague Balázs Kalapos (Department of Biological Resources, ELKH ATK Agricultural Institute, Martonvásár, Hungary). The paired-end reads were trimmed and quality filtered by Trimmomatic v0.36 than FastQC v0.69 were used for general quality controls. The sequencing reads were mapped against the *Hordeum vulgare* L. reference genome (Ensembl Plants release-38) using a HISAT2 aligner v2.1.0 with default parameters. Normalization data transformation protocol has done as the properly mapped reads were counted from the alignment files by the featureCounts tool v1.5.3 then the DESeq2 tool v2.11.39 were used to determine differentially expressed genes (DEGs) from the count tables. Only the gene expressions records which passed the following criterion were used for functional annotations: $p \leq 0.01$ and $-1 \leq \log_2 FC \leq 1$.

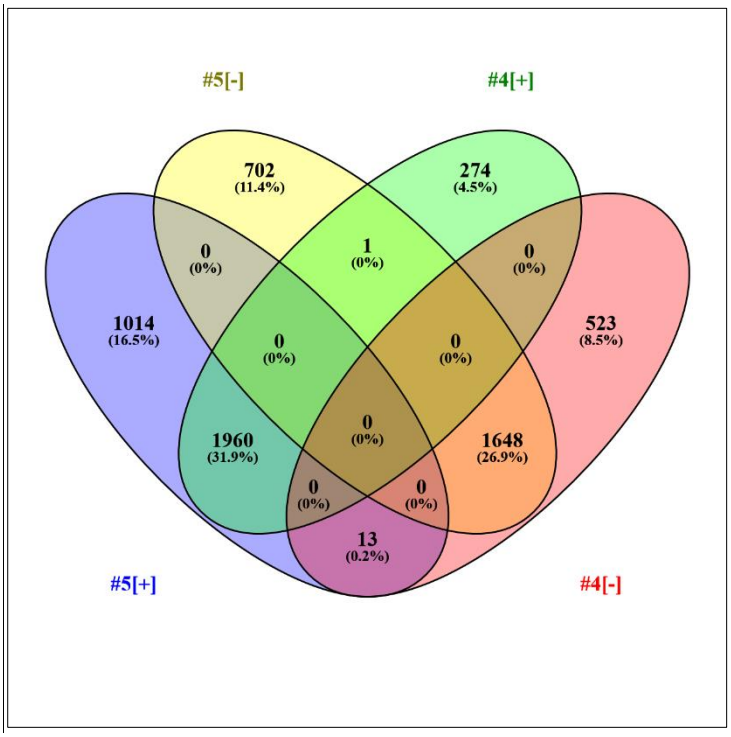
After checking the quality of the given 18 reads from the RNA Seq analyses and fitted them in the barley reference genom available, the expression values were counted. In nine different comparisons, 8142 genes were found so that its expression changed at least double-fold. We have analysed the transcript variants from the same gene as well and we have found some gene where about 30-40 different transcripts were transcribed. They are changed by the time or by the treatment while some of them are showed tissue-specific profile. Genes were annotated functionally using UniProt, KEGG, Pfam and Gene Ontology data bases. Nearly 2000 genes were placed in 124 KEGG metabolic pathways which belong to 18 bigger metabolic classes. The promoter regions of the given 8142 genes were also examined based on whether ABRE or DREB motifs are in them. Recently the interactions/connections between genes and proteins are under analyses based on different network-conceptions.

On the next figures, Venn diagrams summarise our RNA Seq data. With the help of this figures we can get information about the number of differentially expressed genes (DEGs) in response to short-term cold-treatment or the effect of the given CBF transgene, in wild type spring barley and in the CBF transgenic lines.



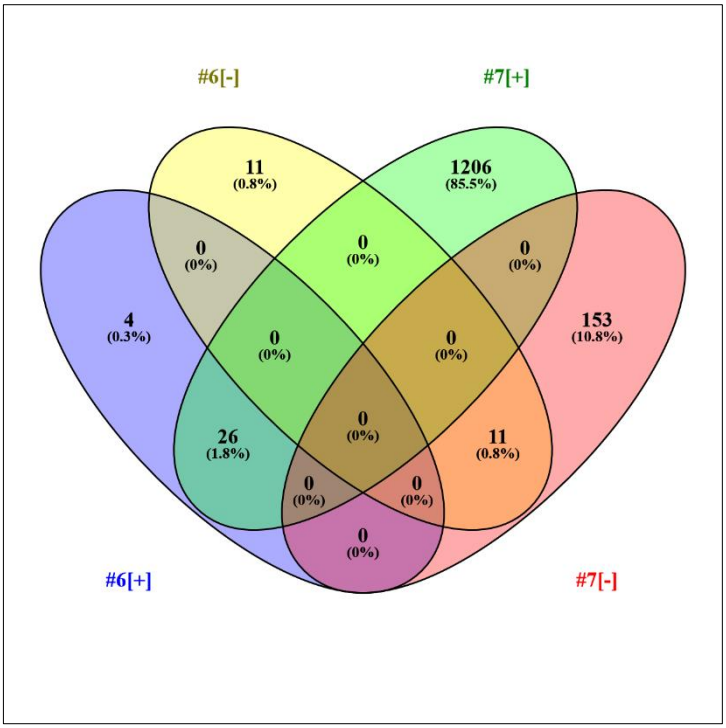






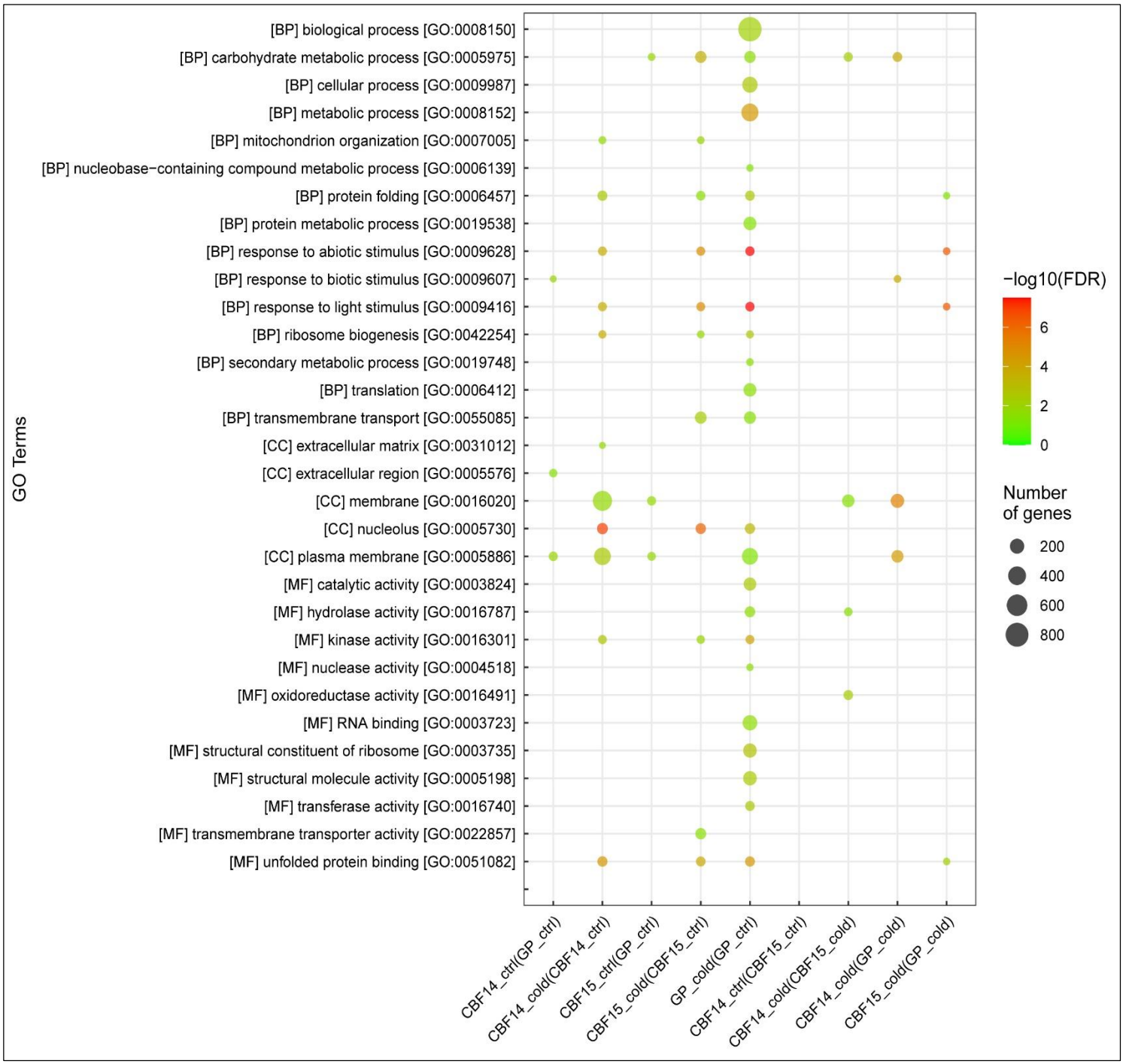
Venn diagrams show the number of differentially expressed genes (DEGs) in response to short-term cold-treatment in wild type spring barley and in the CBF transgenic lines, in different combinations; where number# means:

1#: CBF14 transgenic line control sample versus Golden Promise (GP) wild type barley control sample;
2#: CBF14 transgenic line control sample versus short-term cold-treated CBF14 sample;
3#: CBF15 transgenic line control sample versus Golden Promise wild type barley control sample;
4#: CBF15 transgenic line control sample versus short-term cold-treated CBF15 sample;
5#: Golden Promise wild type barley control sample versus short-term cold-treated GP sample.



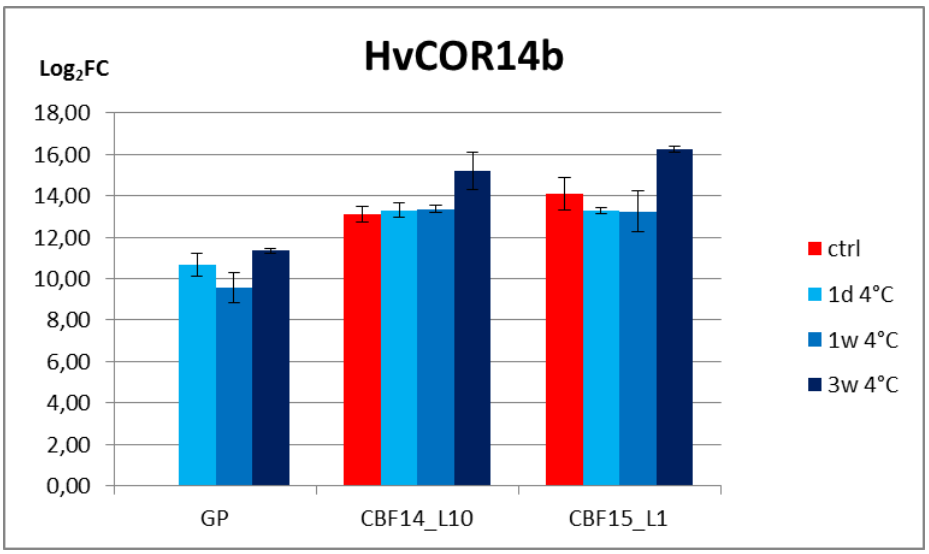
Venn diagram shows the number of differentially expressed genes (DEGs) in response to short-term cold-treatment in the next pairing: **6#:** CBF14 transgenic line control sample versus CBF15 transgenic control sample comparing to the **7#:** CBF14 transgenic line short-term cold-treated sample versus CBF15 transgenic short-term cold-treated sample.

Gene Ontology (GO) enrichment analysis (figure is on the next page) was performed to investigate overrepresented biological processes and molecular functions involved in response to short-term cold-treatment or as the effect of the given CBF transgene. 31 GO term (categories) changed significantly in the samples. 15 of them belong to the 'Biological Function' [BP], 5 terms belong to 'Cellular Component' [CC], 11 GO term belong to the 'Molecular Function' [MF] categories. The size of the points denotes the number of the genes belonging to the given category, the colour of the points indicates the significant level. The analysis was performed by the BiNGO plugin of Cytoscape software. In addition, hypergeometric test and Benjamin & Hochberg False Discovery Rate, $P \leq 0.05$ were employed in the statistical analysis. The 'ggplot2 R-modul' was used to the presentation of the results.



Functional classification of assembled unigenes based on Gene Ontology (GO) categorization.

To validate RNA Seq results and earlier gene expression studies, 26 RNA samples were isolated from the experiment. We have established again the expression level of some known cold-induced genes like HvCBF14, HvCBF12, HvCOR14, HvDHN5 and HvDHN8 by quantitative Real-Time PCR method in these samples. During the unique gene expression studies it was proved again, that, for example, HvCOR14b were enhanced in the wild type Golden Promise spring barley line after cold-treatment; however they are over-produced in the transgenics already in control conditions. The expression of these genes is more induced by cold in transgenic lines.

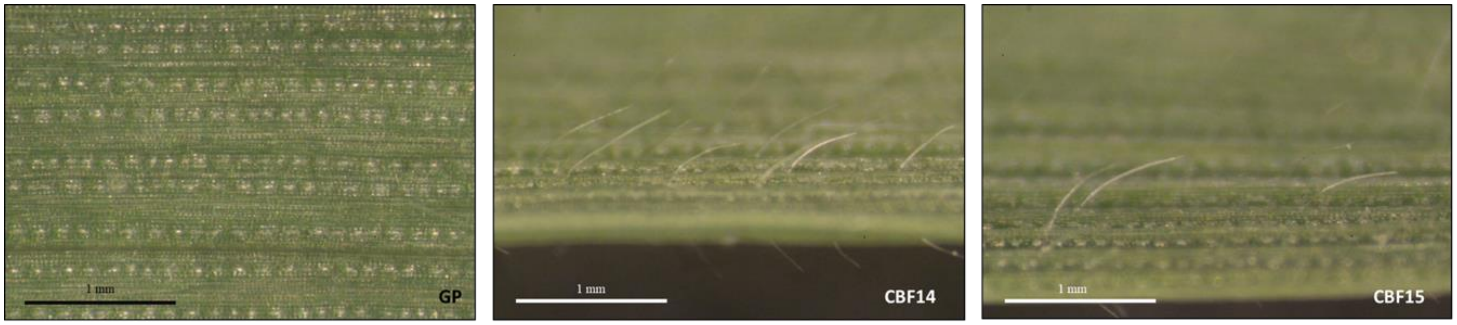


Analyses of the expression level of HvCOR14b confirm the experimental procedure of RNA Sequencing. This gene is up-regulated in the wild type Golden Promise (GP) spring barley line after cold-treatment; however it is are over-produced in the transgenics (CBF14 and CBF15 lines) already in control conditions. The expression level of HvCor14b is more induced by cold in transgenic lines.

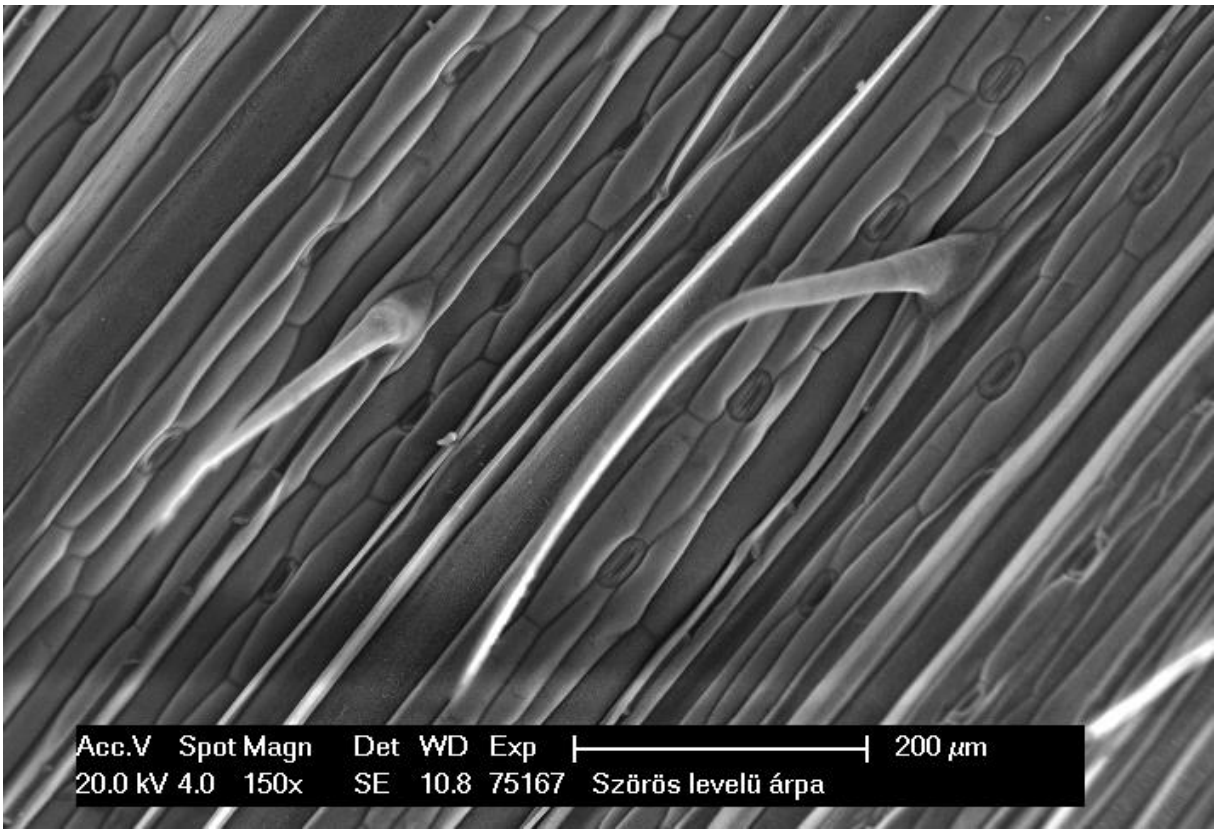
Gene expression analyses were continued with the analyses of some new candidate gene of interest taking the results of RNA Sequencing. Oligonucleotide primer pairs were designed and tested for newly identified target genes belonging to the CBF regulon. I will show these results in the next topics.

Trichomes and CBF genes

During the plant development, a point of interest has been observed in the phenotype of barley transgenics. In the case of wild type Golden Promise barley, the plant leaf has no trichomes, the leaves are without hairs of any kind, and this genotype has glabrous leaf. Also the transgenic control barley line has glabrous leaf. In front of this, TaCBF14 and TaCBF15 overexpressing barley lines have non-glandular trichomes on their leaves, they have pubescent/hairy leaves. It has been documented by light microscope in our institute and with the kind help of Prof. Kristóf Kovács in Pannon University (Veszprém, Hungary) scanning electron microscopically photos were taken too about the trychomes on leaf surface.



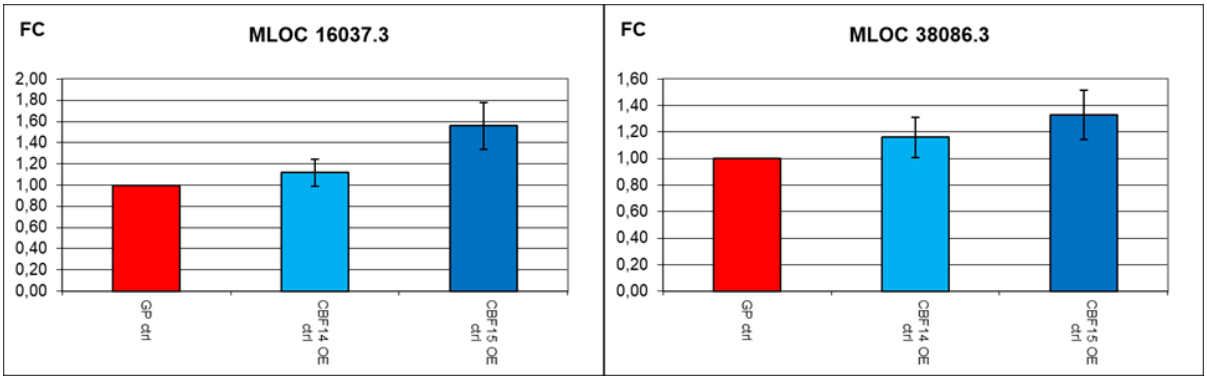
Microscopic analyses of the leaf surfaces of the GP (**GP**: cv. 'Golden Promise' wild type), CBF14 and CBF15 overexpressing barley lines.



Scanning electron microscopical photo shows the structure/shape of trichome of CBF transgenic barley line.

The reason of the development of trichomes were investigated in silico by bioinformatics methods and at gene expression level by quantitative Real-Time RT-PCR. To find connection between the CBF transcription factors and trichome development, gene sequences were analysed in Arabidopsis data base [searched in TAIR database (www.arabidopsis.org), from Gene Ontology Annotations] than in *Hordeum vulgare* data base, to find sequences for "trichome" keyword and about 100 genes were found. Their near homologues sequences in barley were investigated in EnsemblPlants genome database and we

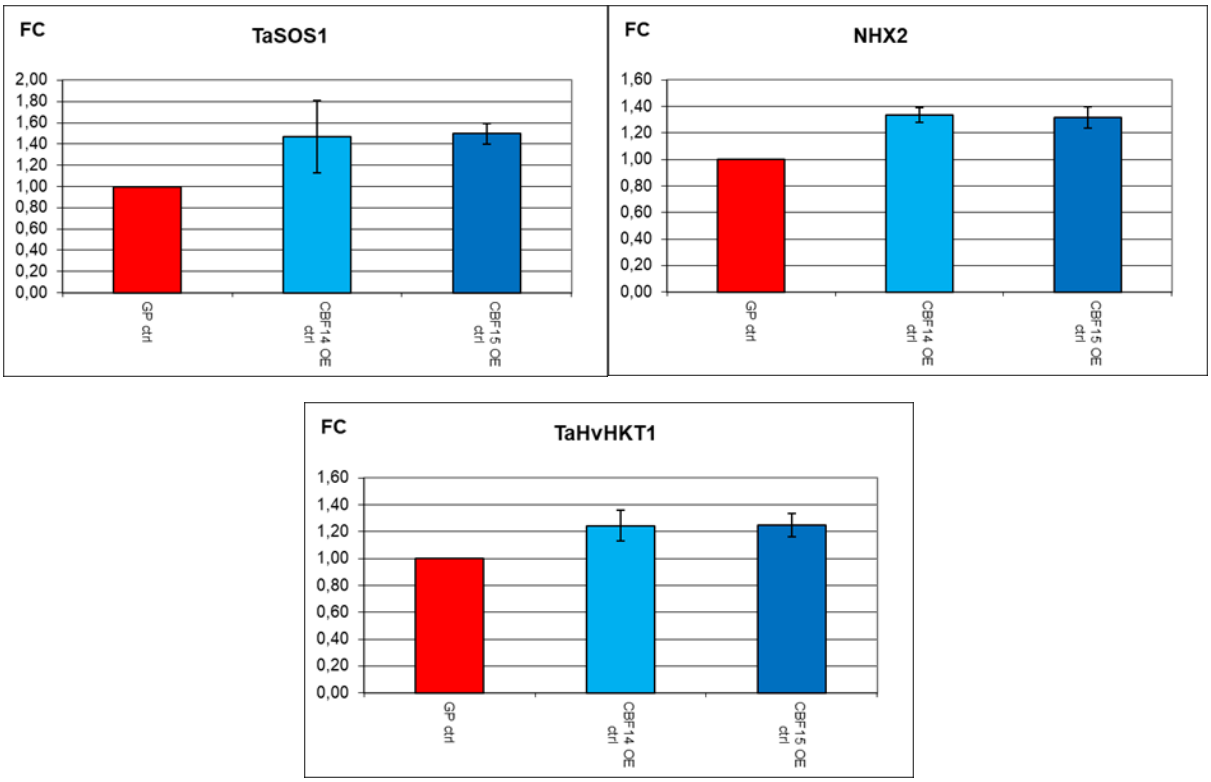
have found 60 target genes. The promoter region of these gene sequences were examined for the 5'-[AG]CCGAC-3' core cis-motif sequence, the CRT/DRE (C-repeat/dehydration responsive element), because CBF transcription factors can bind to the above-mentioned sequences in the promoter of their target genes. Taken the first 500 or 1000 nucleotide from the promoter by the 5' region the results showed 8 or 13 target gene in barley in the connection with trichome development. Primer pairs have been designed and ordered for the 8 most possible target genes, and their expression level were analysed by quantitative Real-Time PCR method. Later we expanded this examination for 25 target genes, and it has been shown that some of them have enhanced expression level in transgenic lines than in the wild type Golden Promise barley. Also the data from RNA Sequencing have been analysed with this object and we have found a sequence which is over-produced in the transgenics, and which sequence is homologue with Arabidopsis EGL3 gene. This is a well characterized gene which have role in the trichome formation in Arabidopsis.



Figures show enhanced expression level of some possible target genes which have CRT/DRE (C-repeat/dehydration responsive element) in their promoter region; in transgenic lines (CBF14 OE – overexpressing- and CBF15 OE) compared to the wild type Golden Promise (GP).

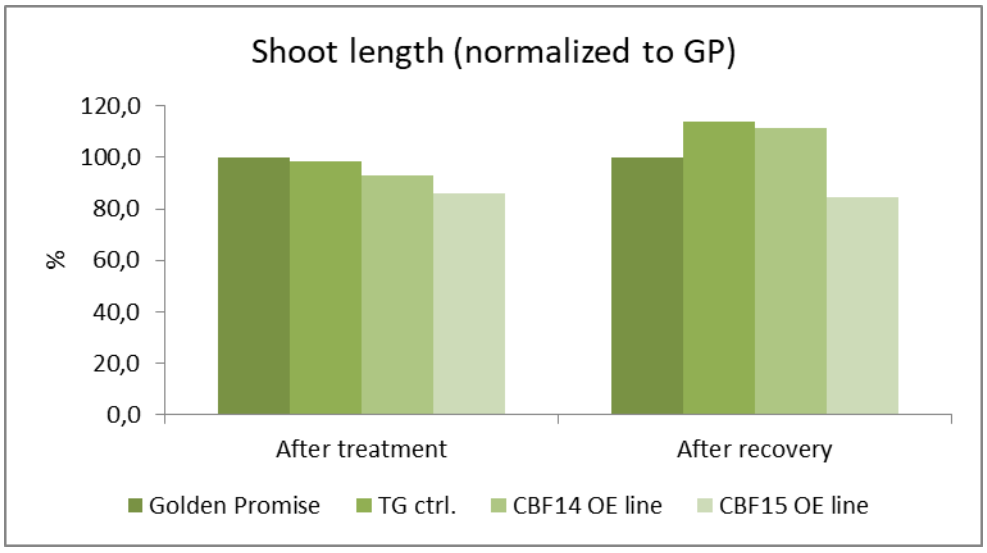
Salinity stress tolerance measurements

As trichomes have role in different abiotic stress tolerance, also in the defence to salinity stress, we have analysed our RNA Seq data in this point of view too. We have measured the expression level of some possible target gene in this aspect by quantitative Real-Time PCR in transgenic lines compared to the wild type Golden Promise barley.

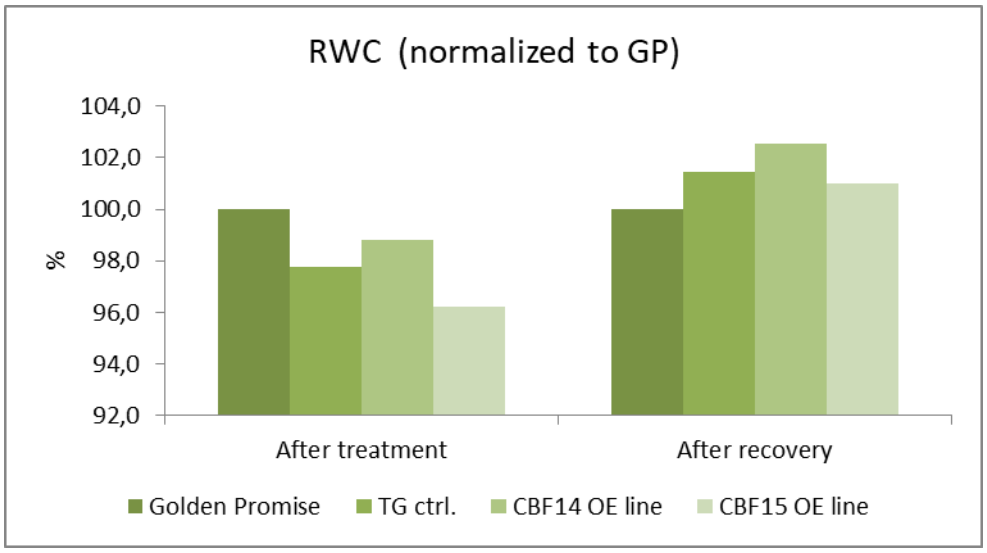


Figures show enhanced expression level of some possible target genes which can have role in the defence to salinity stress. Measurements were carried out by quantitative Real-Time PCR in CBF14 and CBF15 overexpressing (OE) lines comparing to the wild type Golden Promise (GP) barley.

In addition, salt tolerance tests were performed. A preliminary salt-treatment experiment using the transgenic material germs (wild type, transgenic control line and the CBF14 and CBF15 transgenic lines, including the barley and the wheat lines too) implied that transgenic lines have significant salt stress tolerance and improved regeneration capacity after salt stress. To verify this result, we have repeated the experiment using the CBF overexpressing barley lines and the wild type Golden Promise spring barley seedlings; with added sodium chloride in 100mM concentration in a hydroponic system, in plant growth chamber (Phytotron of ELKH ATK Agricultural Institute, Martonvásár, Hungary; in controlled conditions: 20/17°C day/night temperature, 12 h illumination, 400 µmol $m^{-2}s^{-1}$ light intensity, 65% relative humidity). Sampling were carried out before treatment, after 2 weeks NaCl-treatment (from plants grown on control media and on salt treated media) and after 1 week recovery. Length and weight of the shoot and the root as well as the relative water content (RWC) of the leaves were measured during the experiment. For example, enhanced shoot length changes were detected in the case of the given two transgenic lines after recovery period of the salt stress:

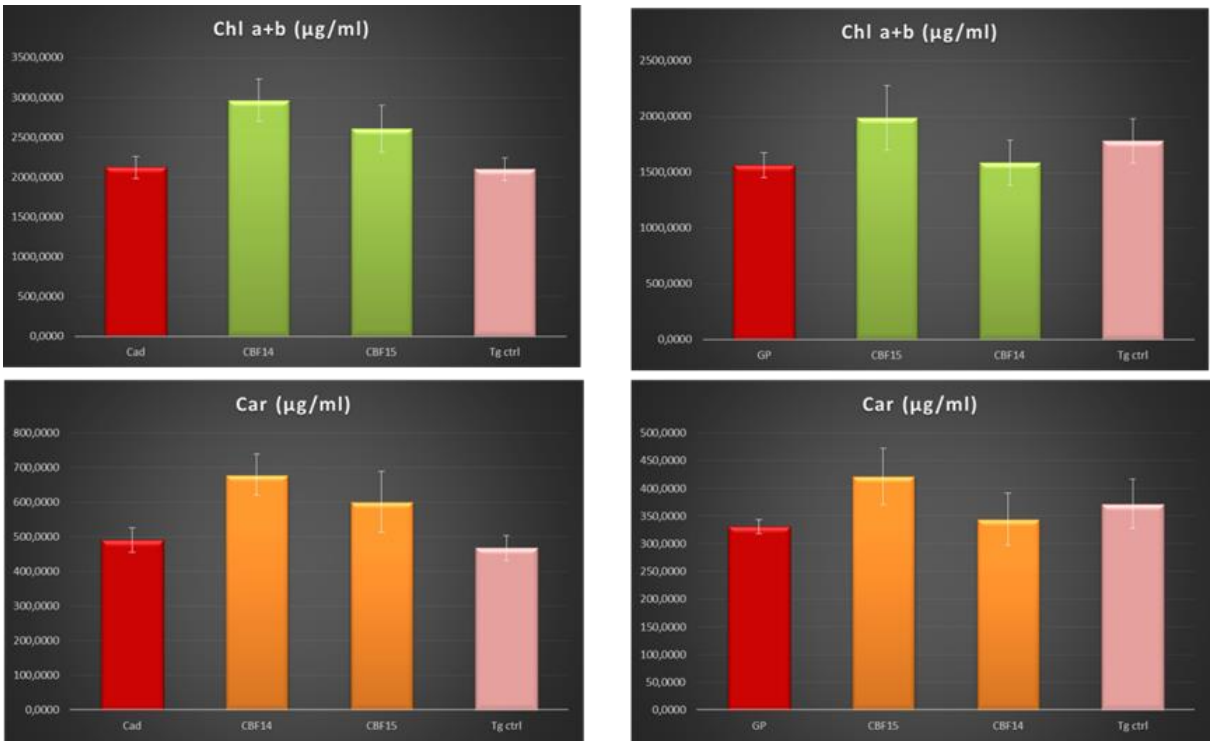


Also the relative water content (RWC) of the leaves were higher after recovery period in transgenic lines:



Chlorophyll a, chlorophyll b and carotenoid content measurements

As we observed differences in the phenotype between the wild type Golden Promise and CBF overexpressing lines in the presence/absence of trichomes, we measured also the chlorophyll a, chlorophyll b and the carotenoid content of the plants by spectrophotometer (Carry100 UV-Vis spectrophotometer, Agilent, USA). The results show that CBF overexpressing barley and wheat lines have approximately 10% more chlorophyll a+b content compering to the wild types. This experiment has been repeated two times with the same results.



Figures show the chlorophyll a+b (Chl a+b) and the carotenoid (Car) content in the wild type spring wheat Cadenza (Cad) and wild type spring barley Golden Promise (GP) and in the two different CBF overexpressing barley/wheat lines (CBF14, CBF15) as well in transgenic control (Tg.ctrl) lines.

Metabolomics analyses and THIOL (glutathione) measurements

In further test, control and cold-treated as well as ascorbic acid (ASA) or H_2O_2 –treated (with 20 mM ASA or 20mM H_2O_2) samples were prepared. From each cases, samples were collected under control conditions and after short-term (3 and 7 days) treatment too, for gene expression studies and for physiological experiments as metabolomics analyses and for THIOL (glutathione) measurements.

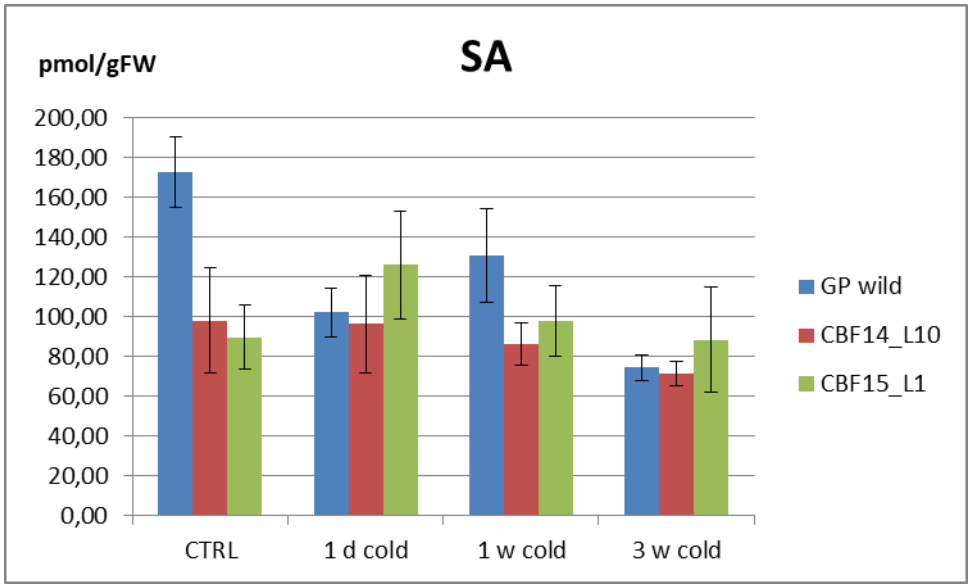
To metabolomics analyses, control, cold-treated and ASA/H_2O_2 –treated samples were explored then transferred to vials and injected to the LECO Pegasus 4D GCxGC TOFMS equipped in split mode, with 30 m column (Rxi-5MS phase) and 1.5 m column (Rxi-17Sil MS phase), and constant flow rate was applied in the comprehensive two dimensional gas chromatographic mode. For the identification, both standards and Kovats retention index were used as well. Both of the GC analyses and data processing were performed by LECO ChromaTOF 4.72 software using LECO-Fiehn metabolomics Library and an up-to-date mass spectral library (NIST). The measurements were carried out with the kind help of my colleague Orsolya Kinga Gondor, Phd (ELKH ATK Agricultural Institute, Martonvásár, Hungary).

The same samples were prepared also for THIOL measurements to study the changes in the redox state of glutathione system. Reduced (GSH) and oxidized (GSSG) form of glutathione were separated by reverse-phase HPLC (Waters, Milford, MA, USA) and detected by a W2475 scanning fluorescence detector (Waters, Milford, MA, USA).The amount of GSH and GSSG

as well the GSSG/GSH ratio increased in the leaf extracts after the different treatment. I am very thankful for the kind help of my colleague Gabriella Szalai, Phd (ELKH ATK Agricultural Institute, Martonvásár, Hungary), who carried out this measurements.

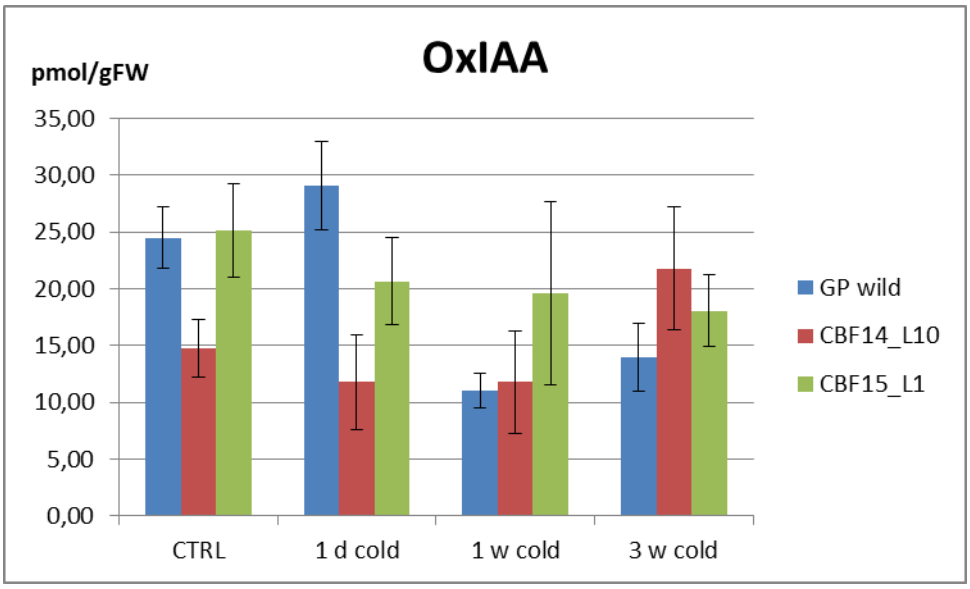
Hormonal examination

Additionally, we have studied the possible linkage between the CBF transcription factors and the hormonal regulatory pathways by an extensive hormonal examination. To this, control and cold-treated (1 day, 1 week and 3 weeks of cold-treated leaf samples) barley and wheat samples (wild type spring barley genotype 'Golden Promise', GP; wild type spring wheat genotype 'Cadenza', Cad; and CBF14 as well as CBF15 overexpressing transgenic lines) were sent to Dr Radomira Vankova (Laboratory of hormonal regulations in plants; Institute of Experimental Botany AS CR, Rozvojova 263, Prague 6, CZ-16502, Czech Republic) for hormonal measurements by HPLC (High-performance liquid chromatography) / MS (mass spectrometry) method. All together the level of 60 different hormone or hormone derivatives were measured. Interest differences were detected at hormonal level in the plant material. For example, lower salicylic acid (SA) level were measured in the transgenic barley lines in control condition compared to the wild type and this value is similar to the value of each sample's SA level after 3 weeks cold-treatment.



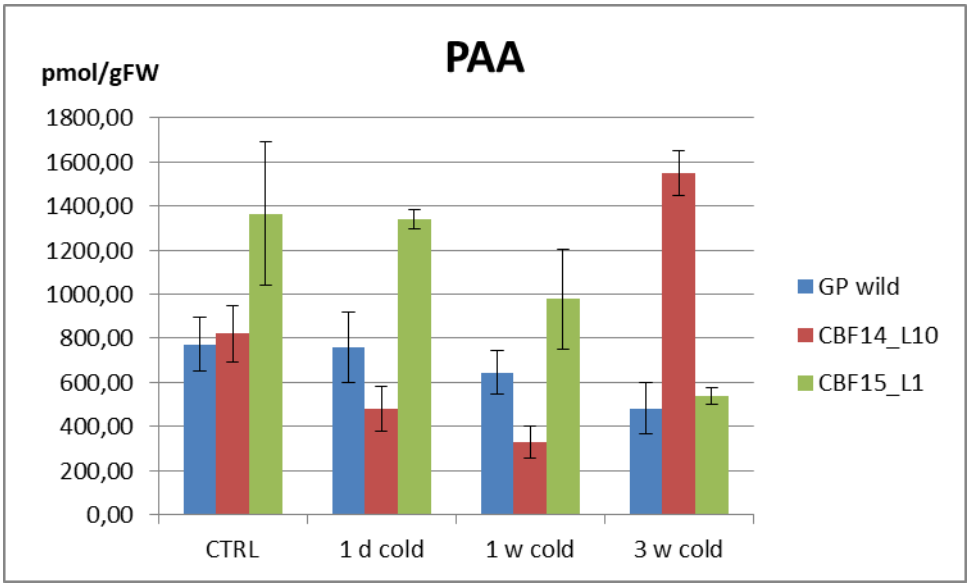
Salicylic acid (SA) level in the transgenic barley lines (CBF14 and CBF15) in control and cold condition compared to the wild type (Golden Promise, GP).

From auxins, the major indole-acetic acid (IAA) catabolite 2-oxoindole-3-acetic acid (oxIAA) level was lower in the CBF14 transgenic barley lines in control condition and after 1day cold-treatment compared to the wild type GP or CBF15 transgenic.



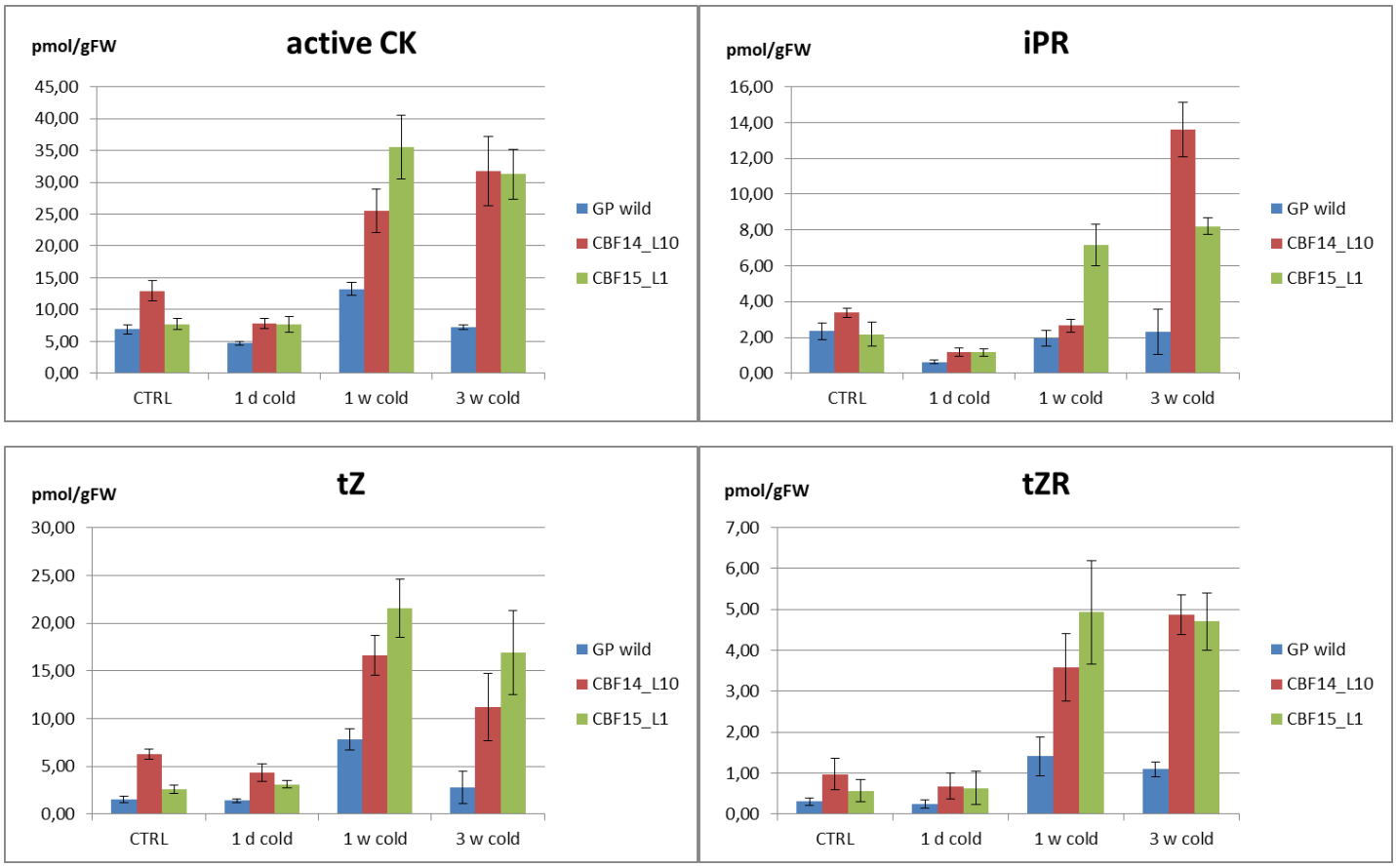
2-oxoindole-3-acetic acid (oxIAA) level in the transgenic barley lines (CBF14 and CBF15) in control and cold condition compared to the wild type (Golden Promise, GP).

In front of this, phenylacetic acid (PAA) level was higher only in CBF15 transgenic barley line in in control condition and after 1day and 1 week cold-treatment compared to the other lines.



Phenylacetic acid (PAA) level in the transgenic barley lines (CBF14 and CBF15) in control and cold condition compared to the wild type (Golden Promise, GP).

From cytokinins, the level of active cytokinins (active CK), trans-zeatin (tZ), trans-zeatin riboside (tZR), and isopentenyl adenosine (iPR) were higher in transgenic lines after 1week and 3 weeks cold-treatment compared to the wild type GP.



Active cytokinins (active CK), isopentenyl adenosine (iPR), trans-zeatin (tZ) and trans-zeatin riboside (tZR) level in the transgenic barley lines (CBF14 and CBF15) in control and cold condition compared to the wild type (Golden Promise, GP).

ORIGINALITY, BASIC RESEARCH AND UTILIZATION

Based on my best knowledge, the effect of an overexpressed CBF gene on the transcriptome pattern has not been studied in cereals yet. The outcomes of the project is relevant from both basic research and practical point of view. Monitoring of the transcriptome profiles of wild type Golden Promise spring barley genotype versus TaCBF14 as well TaCBF15 overexpressing barley lines provides valuable data improving our information on the molecular mechanism of the cold-acclimation and the development of frost tolerance in economically important cereal. The raw data deposited of the RNA Sequencing experiments into ArrayExpress data repository (https://www.ebi.ac.uk/arrayexpress/) will be available for the scientist (it is in progress); with the identifier ‘E-MTAB-10781’.

INTERNATIONAL COLLABORATIONS:

In the course of RNA Sequencing experiment we were in collaboration with Prof. Tamás Dalmay (Head of School, Professor of RNA Biology, School of Biological Sciences, University of East Anglia, Norwich, United Kingdom) and his research group.

Plant hormonal changes were investigated in the collaboration with Radomira Vankova, PhD (Laboratory of hormonal regulations in plants; Institute of Experimental Botany, Prague, Czech Republic).

DIFFICULTIES DURING THE PROJECT

The project was intermitted from 01.02.2018 to 29.02.2020 as I was on maternity leave. During this period I was in connection with my colleagues and we wrote and published our result about the role of up-stream genes in cold-stress response of transgenic barley lines topic in Gierczik at al. 2019 (Plant Molecular Biology Reporter; IF: 1.907). In this article, I am the shared last and corresponding author with Attila Vágújfalvi.

Unfortunately, by reason of COVID19 pandemic, we spent some month in "home office" in 2020 spring (immediately after my maternity leave) and in 2021 spring too. The laboratory experiments were in abeyance at that period. Therefore we summarized the results from our experiments about the molecular background of endodormancy in Populus hybrid cultivars. In this study, several CBF transcription factor expression level were evaluated by quantitative Real-Time PCR method in buds and in leaf samples; as well phytohormones profiles were monitored during growth cessation, cytokinins content and ABA concentration in bud and leaf samples. The article has published in BMC Plant Biology (IF: 3.497) and I am the shared first author with Ákos Boldizsár (Boldizsár et al. 2021).

Also by the reason of COVID19 pandemic, congresses have been cancelled or deferred in 2020 and in 2021. We have written and submitted a conference abstract to an international conference in Riga, Latvia, where we wanted to participate with a poster from the research topic of PD116564 project, but also this congress has been cancelled in 2020.

Writing article from the RNA sequencing and from the processing of the data produced is in progress so I tried to present our experiments abounding in results in detail/thoroughly in this final report.