PhD THESIS

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Investigation of the regulation of the phospholipid-CBF signaling pathway in model plant barley

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1. INTRODUCTION

By 2050, the world's population is prognosed to grow from the current 7.7 billion to about 9 billion, while its food needs will increase by 85% (Raza et al., 2019). Therefore it will be inevitable to continuously increase the annual cropyield. Cereals are among the most widespread and oldest cultivated plant species on Earth, and their role as a raw food material stands out from other crops. In some developing countries, almost only wheat, corn or rice make up the entire diet of the population - so the cultivation is essential, moreover the increasing crop safety can lead to significant economic savings.

The abiotic plant stress tolerance is a result of complex processes, therefore the detailed knowledge in this area is an essetial key to achieve higher crop safety. For decades, the Department of Plant Molecular Biology at the Agricultural Institute, Centre for Agricultural Research has been studying the adaptation of cereals to environmental factors and their molecular background, focusing on the signaling processes responsible for developing low temperature stress tolerance.

During our research, we studied the ‘phospholipid signaling → Ca^{2+} signaling → CBF transcription factors’ pathway and the molecular regulatory mechanisms acting on these elements.

One of the main areas of interest in our research group is studying the molecular mechanisms that lead to the development of low temperature stress tolerance in cereals. In this work, we investigated one of the main components of the increasing cold stress tolerance, namely the gene family encoding CBF (C-repeat binding factor) transcription factors, and also the regulatory mechanisms acting on them. The relationship between the Ca^{2+} signaling pathway and

CBF transcription factors was first demonstrated in the model plant *Arabidopsis*: it was shown that CAMTA3 (calmodulin-binding transcriptional activator 3) can regulate the *AtCBF2* gene (Doherty et al., 2009) proving the role of calmodulins – and thus the Ca^{2+} signaling pathway – in cold tolerance. The phospholipid signaling pathway is partly responsible for the release of Ca^{2+} ions, one of the most well known secondary messenger molecules; but so far few studies have demonstrated the role of the initial genes of this signaling pathway (*PITP* and *PI4K*) in stress tolerance. The prominent role of Ca^{2+} in stress tolerance has long been known; its role in the enhancement of cold tolerance has already been investigated in many plant species.

Apart from the main signal, i.e. the low temperature, the transcription level of the *CBF* genes is influenced by the length of illumination, the intensity, and the spectral composition of the illuminating light as well. Phytochromes are responsible for absorbing red and far-red light. PIF transcription factors are able to interact with these photoreceptors, thus playing an important role in the regulation of different signaling pathways, among them the biotic and abiotic stress induced signaling ones.

2. AIMS

- To investigate the circadian expression of barley *CBF* genes according to their phylogenetic subgrouping, and also the effect of the low red/far-red ratio illuminating light on their gene expression pattern.

- To study which elements of the Ca^{2+} and phospholipid signaling pathways – having influence on *HvCBF* genes – show circadian rhythm and which component(s) are affected by low red/far-red light.

- To investigate the role of two genes, *HvPITP* and *HvPI4K*, involved in the initial steps of the phospholipid signaling pathway by analysis of transgenic plants in the enhancement of barley abiotic stress tolerance.

- *In silico* identification of phytochrome interacting *Hv*PIF transcription factor sequences, their phylogenetic grouping, and identification of the phytochrome binding sites.

3. MATERIAL and METHODS

3.1 Investigation of the circadian gene expression pattern

To investigate the circadian rhythm of the studies genes, we studied a highly frost tolerant autumn barley (*Hordeum vulgare* spp. *vulgare*) variety, i.e. Nure (Genomics Research Center, Fiorenzuola d'Arda, Italy). After standard germination, the plants were grown in wooden boxes for seven days in a Conviron PGV36 (Controlled Environments Inc.) climate chamber with 12 hours of illumination at 20/17°C (day/night temperature) and 70-75% relative humidity. The light intensity was 250 $\mu mol\ m^{-2}s^{-1}$ in the plant growth chamber, which was provided by fluorescent light tubes (Sylvania 215 W F96 T12). Later, the temperature of the plant growth chamber was changed to continuous 22/22°C, while the other environmental parameters were not altered. This period lasted for five days, and by the end of the period the plants reached development stage Z13 (Zadoks et al., 1974), when the wooden boxes were divided into two subgroups inside the plant growth chamber for light treatment. Half of the plants continued to grow under the white light emitted by fluorescent light tubes (same as during the pre-growing period), while the second subgroup received supplemental far-red light in addition to the white light. Reflective separator was placed between the two groups to prevent light contamination in the chamber. In addition to the white light, the supplemental far-red light was provided by 3W LED panels (Shenzhen Justar Electronic Technology Co., Ltd., China) emitting at 735 nm for eleven days. The light treatment reduced the red/far-red ratio to 0.4 - 0.5, while other environmental parameters remained unchanged.

On the eighth day after the light treatment – immediately after turning on the light – we started collecting leaf samples for gene expression studies. Sampling was performed every 4 hours for 4 days. On the first and second sampling days, the plants continued to receive 12 hours of illumination, however, on the third and fourth days, they were further grown under continuous light.

3.2 Gene function studies of *HvPITP* and *HvPI4K* using transgenic plants

In a previous work of our research group, pBract214-*Hv*PITP and pBract214-*Hv*PI4K constructs were introduced into the immature embryos isolated from Golden Promise (spring barley genotype) by the *Agrobacterium tumefaciens* mediated protocol. 15 independent *HvPITP* (phosphatidylinositol transfer protein) and 13 independent *HvPI4K* (phosphatidylinositol 4-kinase) overexpressing transgenic plants were created. During the morphological characterization of the transgenic plants we examined the development of the shoot apex, determined the amount of photosynthetic pigments and phenotyped the individual plants, i.e. earing and flowering time, total plant height, weight of biomass after drying, height weight and grain number of main ear, total grain number and total weight of grains per plants, number of ears per plants, thousand kernel weight too.

The effect of transgenes on frost tolerance was investigated in two types of experiments. In the first one, a cold hardening period (14 days, 5/5°C) was applied before the freezing (at -6°C), while in the second one, after the pre-growing period – without cold hardening – the plants were exposed to frost (-3°C and -5°C) directly. After freezing, the plants were placed into a regeneration chamber

(18/13°C), the leaves were cut, and a three-week regeneration phase was applied to evaluate the regeneration and survival rate of the individual plants (Sutka, 1981).

A combined cold and hypoxic stress tolerance test on the transgenic lines and the wild-type Golden Promise genotype was carried out (Barla-Szabó and Dolinka, 1988). The combined stress was ensured by the simultaneous use of low temperature and hypoxia. After four days of stress treatment, a regeneration period was applied, and then the survival percentage of genotypes and the number of germs with high vigor was determined.

3.3 *In silico* identification of PIF sequences

The latest version (40th edition) of barley proteome available at the time of writing this work was downloaded from the Ensembl Plants (Kersey et al., 2018) website and used for the *in silico* studies. To identify the putative bHLH sequences, the HMMER version 3.0 program (Eddy, 2009) and the so-called hidden Markov-model (HMM) method was used using the HLH domain profile (PF00010) of Pfam (Finn et al., 2016). The predicted bHLH sequences were manually selected, thus reducing the number of repeats and excluding incomplete hits. Sequence alignment was performed using the Clustal Omega (EMBL-EBI) web program (Sievers et al., 2011). After that, WebLogo version 2.8.2 (Crooks et al., 2004) was used to graphically represent the conserved region of the bHLH domain.

Representative elements of the phylogenetic subgroups of *Arabidopsis thaliana* (*At*) and rice (*Oryza sativa*: *Os*) bHLH proteins (Pires és Dolan, 2010), already identified *PIF* (phytochrome interacting factor) sequences from different plant species (NCBI database) such as *At*PIF, *At*PIL

(PIF-like), *Os*PIF, and soybean (*Glycine max*: *Gm*) PIF sequences were also included in the analysis.

Sequence alignments were performed using Clustal Omega (EMBL-EBI) and the MEGA X software (Kumar et al., 2018) as well. Phylogenetic analysis of the sequences was performed using the Simple Phylogeny (EMBL-EBI) Neighbor-Joining method (Larkin et al., 2007), and the phylogenetic tree produced was checked by bootstrap analysis in 1000 replicates (Felsenstein, 1985). For the graphical analyses of the phylogenetic tree structure we used the software package FigTree version 1.4.3 and the MEGA X software.

4. RESULTS

4.1 Circadian rhythm of the phospholipid, the Ca^{2+} signaling pathway and the *HvCBF* genes

The *HvPITP* and *HvPI4K* genes were examined from the phospholipid signaling pathway. They showed a definite circadian rhythm throughout the whole experiment, suggesting proper functioning of the central oscillator. As a result of supplemental far-red light, the *HvPITP* gene expression levels were decreased, and it lost its periodicity under continuous illumination. The *HvPI4K* expression showed phase shift under low red/far-red light, moreover, it was expressed hours earlier.

The expression levels of several genes encoding HvPLC (phospholipase C), HvPLD (phospholipase D) and calcium-binding proteins, which determine – directly or indirectly – the concentration of cytosolic Ca^{2+} levels were also examined. We found that the expression of these genes did not show a uniform pattern.

The barley genome encodes at least twenty *CBF* genes and they can be divided into three phylogenetic subgroups (HvCBF1-, HvCBF3-, and HvCBF4-subgroups). Our studies were performed by studying not all but a representative number of genes from all three subgroups. The HvCBF1-subgroup includes four *CBF* genes, of which the expression patterns of the *HvCBF1* and *HvCBF11* genes were determined. We found that the applied 22°C was not inductive for these genes. Among the members of the HvCBF3-subgroup, the gene expression patterns of the *HvCBF3*, *HvCBF6*, *HvCBF10A*, *HvCBF12*, *HvCBF15*, and *HvCBF16* genes were examined. From these, only the *HvCBF3* and *HvCBF6* genes were induced at 22°C. We found that neither the *HvCBF3* nor the *HvCBF6* genes have

circadian rhythm. From the seven members of the HvCBF4-subgroup, the expression patterns of the *HvCBF2A*, *HvCBF4B*, *HvCBF9*, and *HvCBF14* genes were determined. We observed that the expression of these four genes showed a high degree of similarity. Their gene expression levels reached their maximums 8-12 hours after the light on. In the case of continuous illumination, all four genes maintained their periodicity and showed circadian rhythm. Exposed to low red/far-red light at 12 hours illumination, the maximum values of gene expression levels were further increased and occurred hours earlier in many cases. Continuous illumination and supplemental far-red light decreased their expression levels and they lost their circadian rhythm.

4.2 Analysis of overexpressing *HvPITP* and *HvPI4K* transgenic plants

It has been mentioned in several studies, and Vyroubalová et al. (2011) dedicated a whole review describing the fact that the transformation event is 'disadvantageous' for the plant in many cases, resulting in mainly phenotypic abnormalities. Phenotypic abnormalities were also found among the plants, transformed with the two genes encoding the initial steps of the phospholipid signaling pathway, namely the *HvPITP* and *HvPI4K*, used in the present work. We found that these occurrences were much higher than what we found in our previous work or what is mentioned in the literature. During the development of plants regenerated from calli, we found that some *HvPITP* and *HvPI4K* overexpressing plants were unable to develop ears at all. During the microscopic examination of the shoot meristem, we found that although the vegetative/generative transition occured in the shoot apex, its development stopped at Z45-Z50 developmental stage (Zadok scale).

Seedlings of three transgenic lines from the segregating first transgenic population were observed to be paler than other plants, and some of them were not even able to turn green when they were exposed to light. We also observed that in the homozygous second transgenic generation, a secondary (possibly later a tertiary) spiklet appeared in the inflorescence of some plants, instead of the lateral spiklet, namely the spike showed a more complex morphology/inflorescence.

As a hypothesis we thought that these two genes may play a role in the low-temperature stress signaling process, therefore we performed frost tests to prove it. Two different approaches, based on Sutka (1981) were carried out to investigate the low-temperature stress tolerance of the transgenic PITP and PI4K lines and the Golden Promise wild type. In the first type of experiment, cold hardening was performed after the pre-growing period. We found that PITP L9 and PITP L13 transgenic lines proved to be the most resistant from the studied 27 independent transformant lines (15 PITP and 12 PI4K): nearly twice as many transformant plants survived the applied -6°C frost treatment than the Golden Promise wild type. Of all the examined lines, PITP L9 proved to be the most vital after freezing: 45% of them ($P = 0.041$) were able to regenerate. In the second type of frost test, transformant lines (6 PITP and 4 PI4K) and the Golden Promise wild type as well, were frozen without cold hardening. Plants were treated at -3°C and -5°C for 16 hours directly, and after frost a three-week regeneration period was applied. Frost damage and survival percentage were calculated. We observed that there was no significant difference between the studied transformant PITP and PI4K lines and the Golden Promise wild type at -3°C. We also found that without cold hardening, the -5°C freezing temperature was lethal for all the tested genotypes,

i.e. neither the transformant PITP and PI4K lines nor the Golden Promise wild type survived the applied treatment.

To further investigate the low temperature stress tolerance of the transgenic lines, a hypoxic treatment combined with low temperature during germination was also tested. We found that the applied treatment was almost lethal to the wild type. In contrast, some transgenic lines were less damaged. PITP L4, PITP L15, PI4K L2, and PI4K L5 lines survived the applied treatment almost at the same level; a slight increase in tolerance was found compared to the wild type. After the stress treatment the survival rate of the PI4K L3 line was significantly increased (at P=0.005 level), compared to the Golden Promise genotype, and, despite the hypoxic low temperature stress, 10% of the PI4K L3 transgenic seeds proved to be vital and able to germinate. In addition, the PI4K L3 line not only survived the stress treatment to a greater extent, but the number of large vigorous germs was also increased significantly (P = 0.031).

4.3 Identification of *Hv*PIF encoding protein sequences

Among the studied ones, the far-red light activated phytochrome signaling pathway was examined in more detail. Until now, only some *PIF* genes have been described in a few plant species; furthermore, to our knowledge, no *PIF* sequence has been identified for either bread wheat or barley.

In this work, we performed *in silico* identification of sequences encoding putative PIF proteins in barley. First, we searched for sequences containing the bHLH (basic helix loop helix) motif using the HMM method. We found 183 sequences, and to identify the putative *Hv*PIF ones, a phylogenetic analysis was performed containing the

reference *Arabidopsis*, rice, and soybean bHLH sequences. Based on the structure of the phylogenetic tree we gained, it was found that the barley bHLH proteins can be divided into 25 subgroups, of which the VII(a+b)-subgroup contained 9 individual barley bHLH sequences.

Among these 9 sequences, the putative *Hv*PIF sequences were determined by motif search. We found that 6 barley bHLHs showed a high degree of similarity at their N-terminal region to the *At* APB (active phytochrome B binding) motif of the reference sequence applied, thus suggesting that the barley genome encodes at least 6 PIF sequences.

In the further investigation, we also found that one of these sequences contained the APA (active phytochrome A binding) motif in addition to the APB motif (Al-Sady et al., 2006; Shen et al., 2008) mentioned above. Summarizing our *in silico* results, we hypothesize that only this protein is able to interact (due to the co-presence of APB and APA motifs) with phytochrome B and phytochrome A proteins in the barley proteome, so this is the only *Hv*PIF protein we could identifiy in the barley genome.

5. CONCLUSIONS

The main aims of our work were to study the regulatory mechanisms acting on (1) phospholipid signaling, (2) Ca^{2+} signaling, and (3) CBF transcription factor pathway using the cereal model plant, barley.

In our experiments, we determined the circadian rhythm of genes associated with the above-mentioned pathways using the Nure genotype grown under artificial conditions. In our experiments, we used white light and white light supplemented with far-red light to determine the effect of low red/far-red ratio on gene expression levels.

First, the initial genes of the phospholipid signaling pathway was studied. We found that the *HvPITP* and *HvPI4K* genes have circadian rhythm, and the expression of *HvPI4K* showed phase shift upon supplemental far-red light. In a comprehensive study of genes involved in Ca^{2+} signaling, we observed that they did not show a uniform expression pattern. The expression of some of the genes examined peaked at the end of the evening hours, although they showed low, constant expression under continuous illumination. Supplemental far-red light did not affect the gene expression levels of these genes. Other genes were not affected by either the circadian clock or light treatment. In a third group, gene expression levels were only affected by low red/far-red light, but not by the circadian clock. All these results illustrate well the highly complex system of genes involved in the Ca^{2+} signaling pathway, as they responded to the same conditions with different response mechanisms. This functional divergence may explain how hundreds of calcium-binding proteins encoded in the plant genome (Day et al., 2002) are able to induce such diverse response mechanisms (Ranty et al., 2016) during different environmental effects.

The last ‘participants’ of the examined signaling pathway are the CBF transcription factors. Out of the three phylogenetic subgroups, members from only two (HvCBF3 and HvCBF4) were expressed at the applied 22°C temperature.

Among the *HvCBF*s, members of the HvCBF4-subgroup, which contain the genes that are important for enhancing plant frost tolerance, were expressed. They showed circadian rhythm, and their expression was increased by supplemental far-red light (typical spectrum at twilight) in many cases. Thus, we could summarize that the three ‘experimental variables’ that we studied, namely the circadian clock, the spectral composition of light, and cold temperature, also have influence in the molecular regulation of the studied phospholipid-, Ca^{2+} signaling pathways and the *CBF* level as well. It would be interesting to examine how other cereals – with different frost tolerance levels – respond to changes to the studied molecular- and environmental factors, and how these variables affect plant stress tolerance, such as drought or especially low temperature stress tolerance.

Due to the circadian rhythm of the studied *HvPITP* and *HvPI4K* genes and the significant role of the phospholipid signaling pathway in signal transduction (resulting in the release of different secondary messenger molecules), we also studied the possible role of these two genes in stress tolerance. Using overexpressing transformant barley lines, we found that the *HvPITP* and *HvPI4K* genes contributed slightly to the enhancement of low temperature stress tolerance in barley. In our experiments, however, in many cases we found developmental/morphological abnormalities (abnormal ear development, abnormal lateral spiklet, plants remained in vegetative phase, chlorophyll deficiency) among the transformant lines. According to our theory, due to the divergence of signaling pathways, the overexpression

of early signaling pathway regulatory elements leads to diversified, (severe) negative effects on physiological processes in the transformant plants. It would also be interesting to examine whether transformation of genes encoding the initial steps of other signaling pathways causes similar, common phenotypic abnormalities, thus we could prove our hypothesis. In any case, to increase plant stress tolerancewe we believe that it is much more suitable to overexpress a transcription factor or an effector gene, rather than the initial regulators of a signal transduction pathway.

In this work we demonstrated the effect of low red/far-red light treatment on the expression levels of different genes in many cases. Because our knowledge of the signaling pathways, activated by far-red light, and the mechanisms that regulate them is quite incomplete, we aimed to identify *in silico* the *Hv*PIF transcription factors that could interact with phytochrome proteins in barley. In our work we identified bHLH proteins and classified them into subgroups using phylogenetic methods. Using known PIF sequences and searching for the phytochrome binding motif as well, we were able to identify the sequences that encode barley *PIF* genes. Our results may provide a basis for detailed understanding of the effects of PIF transcription factors on different signaling pathways, other transcription factors, or even individual genes, activated by red and far-red light. Since PIF transcription factors play a general role in the molecular regulations caused by red and far-red light, it would also be interesting to examine the relationship between the geographic origin/occurrence of the plant (tropical, subtropical, Mediterranean, etc.) and the number of *PIF* genes encoded in its genome and correlate it to their functional polymorphisms, and also to search for possible correlation between the number of phytochrome coding

sequences and the number of *PIF* genes with different complexity.

6. NEW SCIENTIFIC RESULTS

1. We have shown that the *HvPITP* and *HvPI4K* genes from the phospholipid signaling pathway have circadian rhythm, and that the *HvPI4K* gene responds to supplemental far-red light with an earlier expression levels.

2. We found that genes involved in the Ca^{2+} signaling pathway do not show circadian rhythms. Their expression pattern can be divided into three subgroups according to their response to the circadian clock, low red/far-red light, or temperature.

3. We have shown that among the barley *CBF* genes, members of the *Hv*CBF1-subgroup are not expressed at 'room temperature' (at 22°C). We demonstrated that the genes of the *Hv*CBF3-subgroup have no circadian rhythm.

4. We demonstrated that without cold induction, only members of the *Hv*CBF4-subgroup have circadian rhythm, and their expression levels are most intense in the late afternoon or in the early evening hours.

5. We have shown that under the influence of supplemental far-red light the genes of the *Hv*CBF4-subgroup responds with higher expression levels in many cases, and, in addition, they are expressed hours earlier.

6. We have shown that the *HvPITP* and *HvPI4K* genes slightly increased the low-temperature stress tolerance of barley.

7. We have found that the overexpression of the initial elements of the phospholipid signaling pathway increases the frequency of certain developmental abnormalities in the transgenic plants.

8. We have determined barley bHLH proteins *in silico*, classified them into phylogenetic subgroups, and finally *Hv*PIF sequences were identified in the barley genome.

7. PUBLICATIONS RELATED TO THIS DOCTORAL DISSERTATION

Articles published in international journals with IF

Gierczik K., Novák A., Ahres M., Székely A., Soltész A., Boldizsár Á., Gulyás Z., Kalapos B., Monostori I., Kozma-Bognár L., Galiba G. és Vágújfalvi A., 2017. Circadian and Light Regulated Expression of *CBF*s and their Upstream Signalling Genes in Barley. *International Journal of Molecular Sciences*. 18, 1828. (IF_{2017}: 3,687)

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Article published in a Hungarian journal without IF

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Scientific presentation

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