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Identification of signaling pathways involved in cold stress by gene expression studies in cereals

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1. INTRODUCTION

As a result of climate change, the frequency of unusual, extreme weather in Hungary has increased, which generates temperature, drought or osmotic stress for corn plants and other crops, thereby causing a significant loss of production and yield reduction. This phenomenon reinforced the need for increased abiotic stress tolerance of cereals.

Based on all this, it can be seen that it is becoming more and more necessary to create cereals that are much more resistant to environmental factors, in our case to significant changes in temperature. Due to the economic importance of cereals, it is very important to understand the process of cold perception, which can help the work of breeders and agrochemists in increasing the winter resistance of these plants. In order to improve the adaptability of crop plants, an important task is to understand the processes that form the plant's frost resistance through the activation of signaling processes through the operation of cold-induced genes, the results of which research serve as information for practical use.

In the temperate zone, overwintering autumn cereals are sown in October and after they emerge, they adapt to the external conditions. The essence of this is that the length of the day and the temperature gradually decrease. This training process lasting several weeks is called cold acclimatization, as a result of which initially frost-sensitive plants become able to tolerate winter frosts. The process of cold acclimatization is very complex, its effectiveness is based on the interplay of a complex signal-receiving, signal-transmitting, and executive network of genetic and biochemical members (molecules), the details of which are not yet fully understood. The question arises as to how a plant can regulate and operate this complex network. Larger temperature fluctuations that occur within a short period of time are important for the cultivation of crops, so it is a task of outstanding importance to understand as thoroughly as possible

the processes that create the frost resistance of different economic plant varieties through the activation of signaling processes through the operation of cold-induced genes, which results can be useful for plant breeding.

There are many publications about the adaptation of cereals to environmental factors and its molecular background, which are responsible for the formation of low temperature stress tolerance, however, there is currently not much information about the signaling processes that are activated after cold stress.

The detailed molecular description of cold acclimation is mostly studied in the plant *Arabidopsis thaliana*, but due to its importance in agriculture, it is desirable to learn about the molecular basis of this process in cereals as well. In cereals, it is known that a decrease in temperature induces a large set of genes; in the first few hours after the cold exposure, these are mostly regulatory transcription factors that are responsible for the activation of subsequent genes. Such are the *ICE-CBF-COR* system (*ICE: Inducer of CBF Expression, CBF: C-Repeat Binding Factor, COR: Cold-Regulated Gene*). The acclimatization processes are controlled by a specific group of genes, the C-repeat binding factors, i.e. the *CBF* genes. These transcription factors use their DNA-binding domain to bind to the C-repeat (CRT) motif (CCGAC) found in the promoter of the genes under their control and regulate their expression under the influence of low temperature. This sequence can be found in many genes induced by cold and drought stress, including *DHN* (*dehydrin*), *KIN* (*cold-induced*), *COR* (*cold-regulated*).

During our research, we studied the "phospholipid signaling → Ca^{2+}, PLC, PLD signaling → *CBF* transcription factors → effector genes (*COR14b*, *DHN5*)" pathway, as well as the molecular regulatory mechanisms affecting them, among the signaling processes responsible for the formation of frost tolerance. We set up two different experiments, where we examined calcium

and phospholipid signaling in the case of barley and *Triticum monococcum* (einkorn wheat) in relation to frost tolerance.

In terms of pharmacology, we used the inhibitors listed in the following table for our genetic and freezing experiments:

Table 1: Overview of the physiological effect of the inhibitor molecules used during the experiments

Inhibitor	**Effect**
Lanthanum chloride	Calcium channel blocker
EGTA	Calcium binder
Ionomycin	Calcium releaser
Neomycin	PLC inhibitor
Ruthenium red	Vacuolar Calcium channel blocker
Mastoparan	Increases intracellular calcium levels
1-Buthanol	PLD inhibitor
U73122	It inhibits PI-PLC activity
Thapsigargin	Calcium pump inhibitor, releases calcium from stores

2. OBJECTIVES

- Investigation of the cold-induced temporal activation of *CBF* transcription factors linked to the *Fr-2* (wheat evening *Fr-A^{m}2*, barley *Fr-H2*) locus in spring and autumn barley and spring and autumn wheat lines, as well as the later induced *HvCOR14b*, *TmCOR14b* and *HvDHN5* effector genes in barley.

- Semi-quantitative PCR and Quantitative RT-qPCR are used to select those *CBF* genes whose induction increases as a result of short-term cold stress.

- Comparison of inhibitors that block individual signaling molecules, determination of their optimal concentration, and then their use for genetic and phenotype studies.

- Pharmacological studies to compare the effects of PLC, PLD, and calcium signaling processes in winter barley and winter wheat lines in gene groups selected based on RT-qPCR regarding cold acclimation and frost tolerance, using different inhibitors.

- Phenotypic test with whole-plant freezing in the case of winter barley and autumn wheat plants, with which the frost resistance is determined after blocking individual signaling processes.

- Monitoring the consequences of cold-induced membrane damage affecting the physiological state of plants in winter barley and winter wheat after cold training and freezing.

3. MATERIALS AND METHODS

3.1. Plant materials

In our experiments, we used einkorn wheat (*Triticum monococcum*) and barley (*Hordeum vulgare*), their frost-tolerant variants are: *T. monococcum*: G3116 and *H. vulgare*: Nure, and the frost-sensitive types used in the first stage of the tests: *T. monococcum*: DV92 and *H. vulgare*: Tremois.

3.2. Determination of expression intensity of *COR14b* and *CBF* genes during cold stress response

For the experiment, we used in frost-tolerant and frost-sensitive forms. After pre-cultivation in a phytotron chamber at 20 °C, some of the 2-week-old seedlings were cold-hardened in a chamber at 4 °C for 24 hours. The control samples remained at 20 °C during this time. Sampling was done at the end of the 2-week pre-training period (20/20 °C) and in the 2nd, 6th, 12th and 24th hours of the training period (4/4 °C). The samples came from the part of the shoot tip above the root of the plants.

RNA isolation was performed using TRIzol and RNeasy Plant Mini Kit (Qiagen), supplemented by the use of DNase set (Qiagen). In our experiments, we used one-step cDNA synthesis using Molony Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega Corporation, Madison, WI, USA) and Oligo(dT)18 (Thermo Fisher Scientific). The synthesis took place in a 3-step temperature cycle (5 minutes at 25 °C, 60 minutes at 42 °C, 15 minutes at 70 °C), then it was diluted to cDNA samples with a final concentration of 25 ng/µl, which served as templates for the semiquantitative and RT-qPCR experiments.

The oligonucleotide primers used during the PCR were also designed for barley and einkorn wheat using the Primer3 program. We designed 10 *CBF*, *COR14b* genes for wheat, and 11 *CBF* genes for barley. In the case of the latter, the primers *COR14b* and *DHN5* were selected for our experiments based on

literature data (Stockinger et al. 2007; Campoli et al. 2009). *HvActin* and *Ta30797* (Paolacci et al. 2009) genes were used as reference or housekeeping genes.

Starting from the 25th cycle of the PCR reaction, sampling was done every 2 cycles, since the amount of DNA synthesized during 25 cycles was not visible on the agarose gel. The amplified DNA samples were detected on a 1% agarose gel containing ethidium bromide.

Based on the results of the semiquantitative PCR reaction, we used the RT-qPCR technique for a more precise analysis of the activation of the selected cold-induced *CBF* and *COR* genes, in the framework of which only the autumn genotypes were used. The RT-qPCR test was performed with an ABI 7500 device and Power SYBR Green reagent (Life Technology) was used to detect the reaction products. To calculate $^{\Delta\Delta}$Ct, the Ct values were exported to Microsoft Office Excel, where we determined the degree of relative expression according to the method of Livak and Schmittgen (2001), the measured gene expression change in the treated samples compared to the control (calibrator) samples.

3.3. Investigating the effect of PLC, PLD and calcium signaling on the functioning of the *CBF-COR* system using inhibitors

Based on the semiquantitative PCR and RT-qPCR results described in point 3.2., the genes were selected, which were further investigated using inhibitors affecting PLC, PLD and Ca^{2+} signaling.

Skipping the spring types, only the autumn barley and einkorn wheat varieties were used for the inhibitor experiments. Young seedlings were treated with mixtures containing 2-2 ml of inhibitors, which affected some phospholipase signaling components (PLC, PLD) and Ca^{2+} levels (Table 1). Under normal conditions, the plants were kept in the inhibitor solutions in 20 °C phytotron chambers for 16 hours, then a part of the plants was transferred to

a 4 °C phytotron chamber for cold training, the other part of the plants was kept in 20 °C control conditions.

Sampling took place at hours 0, 6, 12 and 24 of the training period (4/4 °C). The entire experiment was repeated twice. The samples consisted of the shoot tip and leaf segments of the plants. RNA isolation and cDNA synthesis are described in Section 3.2. was made on the basis of what was described in point.

3.2. described RT-qPCR conditions, the device used and the program differed slightly from the conditions included in the inhibitor experiment, since the samples were examined within a different type of PCR system. The BioRad CFX96 (C1000 Touch Thermal Cycler) PCR device and KAPA Sybr Fast Universal qPCR reagent (Kapa Biosystems) were used for detection. The results of each sample were compared to the results of the same calibrator sample population raised under control conditions and the relative expression level was determined according to the method of Livak and Schmittgen (2001).

3.4. Effects of cold stress-induced PLC, PLD and calcium signaling on freezing resistance, freezing tests

After genetic tests, we performed frost tests to determine whether the changes in the molecular systems had any effect on the phenotype, or whether they influenced the development of frost resistance. Winter barley and einkorn wheat varieties were used for inhibitor and freezing experiments. Two types of freeze tests were performed in a GP200-R4 liquid freezing system (Grant Instruments, Shepreth, England): freezing of leaf segments and freezing of whole plants.

The 5-day-old young seedlings were pre-grown in Hoagland's nutrient solution for 7 days under normal conditions in a phytotron plant rearing chamber. From the 5th day of pre-cultivation, the seedlings were treated with a mixture containing inhibitors for 2 days. From the last (7th) day of the 20/20 °C pre-cultivation, the plants grown in inhibitor-free or inhibitor-containing

nutrient solutions were trained at 4 °C for 2 weeks. The seedlings used as controls were kept in the 20/20 °C chamber during training.

3.4.1. Conductometric frost test, leaf freezing

Freezing of leaf segments was performed by Webb et al. (1994) was performed based on the protocol described by The 1st sampling and the 1st frost test were carried out on the last day of pre-cultivation (day 7) from the plants treated under control conditions of 20/20 °C. These provided the control, i.e. non-hardened samples. During sampling, 4 leaf segments of approximately 1-1 centimeter each, cut from the middle third of the oldest (lower) leaves of 2 mature plants, gave a sample. These were placed in 12 ml sterile falcon tubes. After sampling, part of the samples were not frozen, but shaken for 2 hours with 8 ml of MQ water, these were used to set the baseline, this was considered 100% survival, i.e. the state with the least membrane damage. The maximum ion outflow (strongest membrane damage) was determined by treating the leaf segments from the same plant with liquid nitrogen, then after thawing they were also shaken with 8 ml of MQ water and their conductivity was measured. We took this value as a 0% survival rate.

The tubes to be frozen were placed in the liquid freezing system. Freezing conditions: the samples were placed in a 2 °C freezer, then gradually cooled for 1-1 hour to 0 °C, -2 °C, and then -4 °C to acclimate the plants to temperatures below freezing. At the beginning of the cooling down to -2 °C, a piece of ice of the same size and mass was added to each sample, so that the formation of ice in the samples could begin. The leaf segments of barley plants were frozen at temperatures of -6 °C, -12 °C and -15 °C, while the leaf samples of wheat seedlings were frozen at temperatures of -5 °C, -8 °C, -11 °C and -14 °C, in each case 1 - for 1 hour. After freezing, the samples were regenerated for 30 minutes at 4 °C, after which 8 ml of water was added, kept at room temperature for several hours, shaken, and finally the conductivity of the solutions was

determined with a conductometer device. After shaking, the conductivity of the samples was determined using a MultiSample Conductometer (Mikro KKT, Hungary) in each case from 4 ml of liquid. The amount of ions flowing out, thus the conductivity of the solution, is greater in plants that have suffered tissue death and tissue damage as a result of freezing than in plants that have survived freezing. On the 7th and 14th days of the 4/4 °C training period, the 2nd and 3rd samplings and frost tests were performed. Freezing, regeneration, shaking and conductivity measurements were carried out in the same way as in the case of control samples.

3.4.2. Whole plant freezing

The freezing test was performed in a liquid freezing system after cold training. We removed the nutrient solution from the roots of the seedlings, then placed them in wet filter paper and bags and froze them. The freezing conditions were as follows: placed in the freezer at 4 °C, the samples were continuously cooled to 0 °C and then to the freezing temperatures. Barley plants were frozen at temperatures of -6 °C, -10 °C and -12 °C, while einkorn seedlings were frozen at temperatures of -4 °C, -7 °C and -10 °C, for 1-1 hour in each case.

After freezing, the samples were allowed to thaw at room temperature. We then removed their leaves, leaving only the clumps of bushiness. The latter were planted in plant-growing pots, which contained a 2:1:1 mixture of garden soil, sand and humus. The plants, which were considered as controls during the viability test and to which the degree of regeneration of the frozen seedlings was compared, were kept at 20 °C during the freezing period, and then planted in a manner similar to the above. The regeneration took place for 12 days at 20°C in a climate chamber. The degree of regeneration was recorded every 3 days, when we measured the length of the shoots and characterized them with scoring values on a discrete scale from 0 to 5.

4. RESULTS

4.1. Determination of expression intensity of COR14b and CBF genes during cold stress response

In the experiment, we compared the genetic expression of spring and fall genotypes of barley and einkornn wheat after the 20/20 °C pre-treatment for a short time (1 day) and 4/4 °C cold training. In the case of barley, the expression level of *HvCBF2, HvCBF3, HvCBF5, HvCBF6, HvCBF7, HvCBF9, HvCBF10, HvCBF11, HvCBF12, HvCBF13, HvCBF14* genes, and in the case of wheat, the expression of *TmCBF2, TmCBF3, TmCBF4, TmCBF9, TmCBF10, TmCBF12, TmCBF13, TmCBF14, TmCBF15, TmCBF16, TmCBF17* and *TmCOR14b* genes were compared between the control (20/20°C) and hardened (4 /4°C) in plant samples using semiquantitative PCR and then RT-qPCR technique.

From the 11 barley CBF genes, we selected 6 genes (*HvCBF2*, *HvCBF6*, *HvCBF7*, *HvCBF9*, *HvCBF12*, *HvCBF14*) based on the preliminary PCR results, which were further investigated. Among these, the *HvCBF6* gene was chosen as a negative control for the inhibitory tests, as well as the transcription factors *HvCBF9*, *HvCBF12* and *HvCBF14*, which are activated to a greater extent after cold stress, in addition to the effector genes *HvCOR14b* and *HvDHN5*. Among the 11 tested wheat *CBF* genes, we selected the 9 genes (*TmCBF2, TmCBF4, TmCBF9, TmCBF12, TmCBF13, TmCBF14, TmCBF15, TmCBF16, TmCBF17*) and the *TmCOR14b* gene that showed high expression activity after cold training and examined them in real- time with PCR. To study additional inhibitory signaling processes, we selected the genes *TmCBF9, TmCBF12, TmCBF13, TmCBF14* and *TmCOR14b*.

Based on our results, it can be said that there are *CBF* genes that are not activated even by cold stress, we found genes with a moderate expression profile and *CBF*s that are activated at a high level. In the case of *CBF* genes, an expression intensity can be observed even after 1 hour of cold training, after a

few hours they reach an increasingly high level, and then in some cases a decline is observed, while for other transcription factors their activity level increases even after 12 hours. The activation of the effector gene *COR14b* is not significant after 1 hour of cold treatment, after 6 hours and 12 hours of cold stress, its level increases continuously.

4.2. Investigating the effect of PLC, PLD and calcium signaling on the functioning of the *CBF-COR* system using inhibitors

In the previous experiments, we selected those genes in barley and einkornn wheat that showed the highest gene expression in response to cold training. In addition, we also selected transcription factors that can be considered as negative controls. Based on this, we wanted to investigate the function of the *HvCBF6*, *HvCBF9*, *HvCBF12*, *HvCBF14*, *HvCOR14b* and *DHN5* genes in barley, and the *TmCBF9*, *TmCBF12*, *TmCBF13*, *TmCBF14* and *TmCOR14b* genes in einkornn wheat and their effect on PLC, PLD and calcium signaling processes. The young seedlings were treated with inhibitors (see Table 1), which have a blocking effect on certain details of the signal transmission. In the case of einkornn wheat, it could be established that they reacted much more sensitively to the individual inhibitors used in our experiment compared to barley. After this experience, we did not perform this type of testing of the *CBF-COR* system (in the case of neomycin).

4.2.1. Investigation of calcium signaling

Calcium signaling was influenced in two ways: on the one hand, we increased the amount of Ca^{2+} with ionomycin, which acts on calcium ionophores, and on the other hand, we inhibited the transport of calcium ions with Ca^{2+} -channel blocking lanthanum chloride and ruthenium red, Ca^{2+} chelator EGTA, and thapsigargin, which affects Ca^{2+} -pumps.

Ionomycin has a rather uncontrollable effect on the gene expression. It is likely to change the expression level of any gene family.

Lanthanum chloride, ruthenium red, thapsigargin, used to block Ca^{2+}-channels, did not affect the expression of *TmCBF13*, which is otherwise low during cold stress, while the Ca^{2+} chelator EGTA slightly increased gene expression. The expression of *Tm*CBF9 and *TmCBF14* was high in seedlings grown under inhibitor-free conditions and their levels were not changed by the inhibitors. In the case of *TmCBF12*, it can be said that each inhibitor slightly reduced its expression. Only thapsigargin had the opposite effect, i.e. it increased gene expression. The level of *TmCOR14b* effector gene expression under normal conditions was of the order of 100. As a result of the treatments, we experienced a small increase. The EGTA treatment proved to be the most effective, as this treatment doubled gene expression.

In the case of barley, lanthanum chloride, ruthenium red, thapsigargin, used to block Ca^{2+} -channels, and the Ca^{2+} chelator EGTA did not affect the expression of *HvCBF6*, used as a negative control. The expression levels of *HvCBF9* and *HvCBF14* were high in barley seedlings grown under inhibitor-free conditions, especially in the case of *HvCBF14*. The expression kinetics of these genes were significantly affected by the inhibitor treatments. *HvCBF9* activation was reduced by all Ca^{2+} pathway blocking substances except for EGTA: lanthanum chloride caused an eight-fold lower expression level under the influence of ruthenium red. The function of *HvCBF14* was reduced by all treatments: EGTA by 1.5 times, other calcium signaling blocking substances by 7-8 times. In the case of *HvCBF12*, it can be said that - apart from EGTA and thapsigargin treatment, for which no significant changes were observed - all inhibitors increased the level: lanthanum chloride doubled, while ruthenium red showed an eightfold increase in expression. The basic level of *HvCOR14b* in the control barley seedlings was of the order of 10,000, except for thapsigargin,

we did not observe any major changes due to the treatments. Thapsigargin, on the other hand, increased gene activity fivefold in the case of *HvDHN5*. Similar to the *HvCOR14b* gene, the effect of the lanthanum chloride and EGTA treatments was negligible and the baseline level was not very high in the case of *HvDHN5*.

In our experiment, mastoparant was used to increase the intracellular Ca^{2+}-level. The level of *TmCBF13* increased ninefold, the function of the *TmCBF9* and *TmCBF14* genes was also positively affected by mastoparan treatment, the activation of *TmCBF9* increased 3-fold, the level of *TmCBF14* increased 2-fold. In the case of *TmCBF12*, the level of gene activity remained unchanged. The expression level of the *TmCOR14b* effector gene increased 2.5-fold. In the case of barley, it can be said that the activation kinetics of *CBF* transcription factors is similar to what has been experienced so far. In the case of *HvCBF6*, the change after treatment is negligible. The operation of the *HvCBF9* and *HvCBF14* genes is negatively affected by mastoparan treatment compared to their baseline level: in the case of both genes, the activation decreased by one and a half times. In the case of *HvCBF12*, the gene function is unchanged. The expression changes of the two effector genes after mastoparan treatment were slightly different. The level of *HvCOR14b* remained unchanged, while the *HvDHN5* gene was positively affected by the treatment and its expression increased 2-fold.

4.2.2. Investigation of phospholipase C (PLC) signaling

In our experimental system, we used U73122 and neomycin inhibitors to inhibit PLC-dependent signaling.

The results of the *Triticum monococcum* genetic test and frost test revealed that the neomycin inhibitor proved to be too toxic, both in terms of the functioning of the *CBF-COR* system and the phenotype. Since it caused confu-

sion in the expression of transcription factors and effector genes, I will not report these results in the dissertation.

In the case of einkornn wheat, after the U73122 treatment, it can be said that the kinetics of the activation of *CBF* transcription factors was as follows: the level of *TmCBF13* was increased fourfold by the treatment. The function of the *TmCBF9* and *TmCBF14* factors was slightly positively affected by the treatment, but to a negligible extent. For *TmvCBF12*, U73122 treatment did not alter gene expression. The examined effector gene was positively influenced by the substance blocking the PLC-dependent signaling pathway, i.e. it increased the level of *TmCOR14b* by 3.5 times.

In barley, following U73122 and neomycin treatment, the activation kinetics of *CBF* transcription factors were similar to those observed in calcium signaling studies. *HvCBF6* levels remained unchanged. The function of *HvCBF9* and *HvCBF14* was negatively affected by the treatment, the expression of *HvCBF9* was reduced by half, while the level of *HvCBF14* was reduced by 6-7 times by both treatments. In the case of *HvCBF12*, U73122 increased gene expression 10-fold, neomycin had no effect on its function. The effector genes were affected differently by the two substances blocking the PLC-dependent signaling pathway compared to the transcription factors: the level of *HvCOR14b* was not affected by U73122, but it was significantly reduced by neomycin, while *HvDHN5* expression was only slightly reduced by neomycin and 6-7 times by U73122 treatment increased.

4.2.3. Investigation of phospholipase D (PLD) signaling

The inhibitor 1-buthanol was used to inhibit PLD-dependent signaling. In the case of einkornn wheat, it can be said that the gene expression kinetics of *CBF* transcription factors did not reflect what has been experienced so far. The level of *TmCBF13* increased two-fold after treatment. The function of the *TmCBF9* and *TmCBF14* genes was also positively affected by the 1-buthanol

treatment, however, the expression of *TmCBF9* was affected only after the 24th hour, increasing it 5-fold. The expression of *TmCBF14* was only slightly increased by the inhibitor intervention, while it was decreased in the case of *TmCBF12*. After blocking the PLD signaling process, the treatment reduced the expression level of the *TmCOR14b* effector gene we investigated by half.

In the case of barley, the expression kinetics of *CBF* transcription factors was as follows: the level of *HvCBF6* was slightly affected by the treatment. The function of *HvCBF9* and *HvCBF14* genes is negatively affected by 1-buthanol treatment compared to their baseline level, i.e. *HvCBF9* expression decreased by one and a half times, and the level of *HvCBF14* decreased by 3.5 times. In the case of *HvCBF12*, gene activity increased 3-fold. Both the level of *HvCOR14b* and the *HvDHN5* gene were positively affected by the treatment, in both cases the gene expression increased by 2.5-3 times.

4.3. Effects of cold stress-induced PLC, PLD and calcium signaling on freezing resistance, freezing tests

Based on the freezing results, ionomycin, thapsigargin, mastoparant, ruthenium red and U73122 were selected due to their excessive toxicity. So, I present the freezing results of plants treated with lanthanum chloride, EGTA, neomycin and 1-buthanol.

For *T. monococcum*, whole plant freezing was lethal at all freezing temperatures. It was concluded that a long-term inhibitory test could not be used, since wheat in its early stages reacted more sensitively to individual substances compared to barley. For this reason, we do not present the results of freeze tests after blocking the individual signal transmission processes. We experienced something completely different in the case of barley, where the treatments did not affect the development of the plants to such an extent, but the physiological effect of some inhibitors was evident in terms of the development of frost tolerance.

4.3.1. Whole plant freezing in the case of barley, effect of inhibitors

After the frost test, we gave the barley plants an individual rating value and determined the survival rate. The survival rate after freezing at -6 °C was still 100% for all treatments, then decreasing the freezing temperature saw fewer and fewer plants survive. After the frost test at -10 °C, the individuals died. After the inhibitor treatment, we found that lanthanum chloride and neomycin had a toxic effect even at 20 °C.

For 80% of plants treated with untrained lanthanum, even freezing at -6 °C was lethal, even freezing at -6 °C was lethal, and in the case of untrained EGTA-treated plants, the entire population froze. After training, the survival rate increased for both control and Ca^{2+} blocked seedlings. Most of the untrained plants did not survive freezing at -10 °C, the survival rate increased only slightly after 1 week of training. The survival rate of plants frozen at -12 °C was similar to those frozen at -10 °C.

The temperature at which relevant results were obtained was -6 °C, which means that the observed survival data are suitable for comparing the effect of individual inhibitors. Even after 1 and 2 weeks of training, -10 °C and -12 °C can be considered lethal, as both inhibitor-free and inhibitor-treated seedlings froze. The treatment with lanthanum chloride, EGTA and neomycin reduced the degree of hardening, while 1-buthanol did not affect the degree of frost tolerance to a greater extent compared to the untreated samples.

4.3.2. Leaf segment freezing in case of barley

The level of ion outflow correlating with the degree of tissue (membrane) damage of the leaves caused by freezing was determined by conductance measurement. During the 2-week training, neomycin and 1-buthanol treatments slightly changed the plants phenotypically and caused tissue changes. Lanthanum chloride and EGTA treatment did not cause significant tissue and

growth disorders. In addition, neomycin and 1-buthanol also greatly influenced the conductance value of the barley plants, even before freezing, there was a difference in conductance in the individuals compared to the control plants grown on Hoagland (its level increased). For these reasons, we only considered the ion efflux of plants treated with lanthanum chloride and EGTA, which block calcium signal transduction, since probably irrelevant results were obtained in the case of plants treated with neomycin and 1-buthanol.

The conductance parameter differed slightly in the case of samples from plants grown under control conditions and samples from trained individuals. Freezing at -6 °C had no significant effect on the conductance level, although a slight increase in its value can be observed as the temperature decreases. The leaves of barley plants frozen at -12 °C were already damaged, the relative ion efflux level increased in these samples, but not to a large extent. As a result of freezing at -15 °C, the ion outflow value approached the maximum level.

In the case of individuals treated with lanthanum chloride and EGTA inhibitors, no significant significant difference in ion efflux compared to untreated plants was observed either in barley grown under control (20 °C) conditions or in hardened individuals, although EGTA treatment increased this value to a small extent. After freezing, a slight increase in ion efflux can also be observed in samples treated with EGTA compared to barley grown in inhibitor-free nutrient solution.

After freezing the leaf segments of the 1-week-old cold-treated individuals at -6 °C, a small increase in ion efflux can be observed at -12 °C and -15 °C, compared to the values measured in plants grown under normal conditions, both In the case of individuals raised on Hoagland medium and kept on nutrient solutions containing inhibitors. The latter temperatures can already be considered lethal. After the two-week training, we froze our barley plants grown on inhibitor-free and inhibitor-containing nutrient solutions at -6 °C, -12 °C and

-15 °C in order to determine the temperature at which the growth of plants' frost resistance can be clearly distinguished, and the effect of individual inhibitors can be easily detected. Based on our results, it can be said that the fitness level of inhibitor-free and treated samples that survived 1 and 2 weeks of training also increased significantly after freezing at -6 °C. As the freezing temperature decreased, the extent of frost damage was greater, and the results correlate with freezing results for whole plants.

Freezing at -12 °C and -15 °C was considered lethal in the case of untrained samples, after the training the degree of frostbite was reduced by half and mostly 2 weeks of training is required for cold acclimatization. In the case of freezing at -6 °C, it can be seen that lanthanum chloride caused a small disturbance in cold acclimatization, and EGTA to a greater extent.

5. CONCLUSIONS

Based on our results, it can be said that there are *CBF* genes that are not activated even by cold stress, we found genes with a moderate expression profile and *CBF*s that are activated at a high level. Our experiment also proved that in the case of cereals, the *CBF14* gene is the most effective and important in terms of developing frost tolerance (Novák et al. 2017), however, in the case of barley, *HvCBF9* and in wheat, *HvCBF12* may also play an important role. It can be said that the function of the effector gene *COR14b* is essential in the cold acclimation processes of both types of grain, and the degree of its expression is particularly important, since a positive correlation was shown between the degree of accumulation of the *COR* gene product and the developed frost resistance (Vágujfalvi et al. 2000).

The conclusion can be drawn from our research that the training in the system we set up was effective, the frost resistance of the cultures increased compared to the control grown at 20 °C, and the inhibitors caused changes in the expression of some *CBF* and effector genes, which confirms what previous research has shown proposed hypothesis, according to which signaling processes are activated during cold stress (Uemura and Steponkus 1999).

Based on freezing tests on wheat lines, we found that wheat plants in their frost-tolerant form are not suitable for pharmacological testing, these plants are more sensitive to inhibitors, so this type of testing cannot be used for them. This kind of sensitivity can probably be explained by the fact that the tissue structure of diploid wheat is similar to that of diploid barley and they are also genetically similar, however, they react much more sensitively to the substances used in the experiments and, in terms of their frost resistance, freeze more easily than barley plants (Vágújfalvi et al. 2003). and this effect only increases with inhibitor treatments.

It can be said that cereal *CBF* genes can be classified into 3 groups according to their expression kinetics in a pharmacological approach: we can distinguish a group of genes that do not react to a large degree after cold stress or to inhibitors, such as *TmCBF9*, *HvCBF6*. The second group of genes includes those *CBF*s that are activated at a high level after cold exposure and this gene expression is mostly reduced by inhibitors, such as *TmCBF12*, *TmCBF14*, *HvCBF9* and *HvCBF14*. The members of the third *CBF* group include those genes that showed a moderate increase in expression after exposure to cold. This included *TmCBF13*, *HvCBF12*. In the case of barley and wheat *COR14b*, it can also be said that it reacted to the inhibitors in different ways, which suggests that in addition to CBF-regulated activation, several other processes can influence its function, such as the length of illumination, the quality of light, and factors related to chloroplasts (Crosatti and et al. 1999, Franklin and Whitelam 2007, Novák et al. 2016). In the case of the barley *HvDHN5* gene, it can be said that the investigated metabolic processes can influence the gene function in a more regulated manner, since almost all blocking with inhibitors increased its expression. This suggests that the calcium, phospholipase C, and phospholipase D pathways also negatively regulate gene expression during the cold stress response. Our results also prove that for the maximum activation of the *COR14b* and *DHN5* genes, in addition to the appropriate expression level of the CBF genes, other factors are also necessary, including the proper functioning of the investigated signaling processes.

After freezing leaf segments, we found that -12 °C and -15 °C can be considered lethal in the case of untrained samples, after training the extent of freezing was reduced by half, mostly 2 weeks of training is required for cold acclimatization. -10 °C is also considered lethal. After 2 weeks of training, the degree of frost tolerance is sufficient for the individuals to survive freezing to -6 °C.

The inhibitor molecules used for the frost tests had different effects on the phenotype of the plants used in the experiment. The lanthanum chloride treatment resulted in a small growth reduction and stem-leaf darkening in the two-week-old barley plants. This can be explained by the fact that it inhibits the curvature growth of the stem to a greater extent (Friedman et al. 1998). According to this, the Ca^{2+}-level reduced by lanthanum chloride has an effect on gravity-related stem elongation processes. We verified this effect in our experimental system. The small amount of leaf darkening can be explained by the fact that lanthanum can influence the plant pigment content (chlorophyll content) and the morphology of the chloroplast (Hu et al. 2016). In the case of the Ca^{2+} chelator EGTA, which also affects the level of Ca^{2+}, we could observe a similar phenotypic change in the plants, but to a lesser extent than in the case of lanthanum, and the darkening of the leaves and stems was also absent. According to this, EGTA can affect elongation processes in the same way as lanthanum and has been proven to affect the functioning of photosynthesis (Tang et al. 2019). In the experiment, using the neomycin inhibitor, the 2-week-old plants became almost lethal, leading to necrosis (tissue death). The treatment resulted in reduced growth, inhibition of leaf and stem elongation, necrosis of root lateral branches, changes in pigmentation (yellowish-brown discoloration), and a morphological phenomenon similar to a dehydrated state could be observed. Neomycin also affects the functioning of chloroplasts, which explains tissue and organ deaths (Teemu et al. 1989). It can be said that phospholipase C-dependent signaling, which is inhibited by neomycin, can be an essential process for the proper development of plants. The roots and their hairs of plants treated with 1-buthanol were shorter and often branched, cell elongation of the roots was inhibited, stunted growth of leaves, yellow-brown discoloration, and modified cotyledon morphology could be observed. *Arabidopsis* studies revealed that 1-buthanol disrupts the organization of microtubules during the interphase of cell division (Gardiner et al. 2003). buthanol isoforms that have no effect on PLD-dependent PA production (2- and

3-buthanol) leave germination, seedling growth and microtubule organization undisturbed. Considering the latter, it can be concluded that phospholipase D-dependent PA production is probably important in the process of root and leaf development.

Summing up the results obtained during the pharmacological tests, it can be concluded that the treatments do not have a drastic effect on the plant *CBF* genes, as well as on the *COR* and *DHN* effector genes, but they do modify them. We can state that the signal transmission systems are able to respond to the cold effect regardless of the external treatment, the difference is in the degree of the response. Since the multitude of gene expression changes regulated by the activation of phospholipases and signal transduction pathways in plants is still an unexploited research area, further studies are necessary to more precisely explore the regulatory network, on which our results can serve as a basis.

6. NEW RESULTS (THESES)

1. We have shown that not all of the 11 barley *HvCBF* and 11 einkorn wheat *TmCBF* transcription factors located on the *Fr-2* (Frost resistance) locus of chromosome 5 that we examined can be considered as cold-inducible genes, which means that the membrane structure change that occurs after a short exposure to cold and the multitude of signaling processes activated by this do not regulate all the *CBF* genes mapped to the frost resistance locus. We showed that there are *CBF*s that are not activated by cold stress (*HvCBF6*), we found genes with a moderate expression profile (*TmCBF13, HvCBF9*, *HvCBF12*) and *CBF*s that are activated at a high level (*TmCBF12*, *TmCBF14* and *HvCBF14*).

2. We showed that among the barley *CBF*s, *HvCBF9, HvCBF12* and *HvCBF14* proved to be cold-activated genes with higher expression after 6 hours of cold exposure. Among the examined wheat transcription factors, *TmCBF12* and *TmCBF14* showed expression activity with similar kinetics after 6 hours of cold stress.

3. In our experimental system, we proved the connection between phospholipase C, phospholipase D and calcium signaling processes with the functioning of *CBF* and effector genes. We proved that, among the inhibitor molecules used, neomycin, which affects phospholipase C signaling, causes a disturbance at the gene expression level in einkornn wheat. Based on freezing results, we showed that the Ca^{2+} ionophore, Ca^{2+} releasing ionomycin, the Ca^{2+} -pump inhibitor thapsigargin, the intracellular Ca^{2+} -level-increasing mastoparan, the vacuolar Ca^{2+} channel blocker ruthenium red and the PI-PLC activation inhibitor U73122 damages it drastically.

4. Cereal *CBF* genes can be classified into 3 groups according to their expression kinetics in a pharmacological approach: we can distinguish a group of

genes that do not react to a large degree after cold stress and to inhibitors, such as *TmCBF9*, *HvCBF6*. The second group of genes includes those *CBF*s that are activated at a high level after exposure to cold and this expression level is mostly reduced by inhibitors, such as *TmCBF12*, *TmCBF14*, *HvCBF9* and *HvCBF14*. The members of the third *CBF* group include those genes that show a moderate increase in expression after exposure to cold. These include *TmCBF13*, *HvCBF12*.

5. Our results prove that for the maximum activation of the effector genes *COR14b* and *DHN5*, in addition to the appropriate expression level of the *CBF* genes, other factors are also necessary, including the proper functioning of the investigated signaling processes.

6. We showed that among the investigated *CBF*s there are genes independent of PLC, PLD and calcium signaling (*HvCBF6*, *TmCBF9* and *TmCBF13*), transcription factors dependent on PLC and calcium signaling (*HvCBF12*), genes dependent on all three signaling (*HvCBF9*, *HvCBF14*, *TmCBF12*), or a gene that is regulated only by certain calcium signaling components (Ca^{2+} -channels, Ca^{2+} -level) (*TmCBF14*). *HvCOR14b* expression depends on some PLC and calcium signaling components. *HvDHN5* expression depends on some calcium signaling components, but it functions independently of PLC and PLD signaling. *TmCOR14b* gene function depends on phospholipase D signaling.

7. After freezing a barley leaf segment, we found that -12 °C and -15 °C can be considered lethal in the case of untrained samples, after training the rate of freezing was reduced by half, mostly 2 weeks of training is required for cold acclimatization. Based on the frost test of the whole plant, it can be said that even -10 °C is considered lethal. After 2 weeks of training, the degree of frost tolerance is sufficient for the individuals to withstand freezing to -6 °C.

PUBLICATIONS RELATED TO THE PRESENT STUDY

Articles published in international journals related to the topics presented in the dissertation:

Zsuzsa Marozsán-Tóth, Ildikó Vashegyi, Gábor Galiba, Balázs Tóth (2015) The cold response of CBF genes in barley is regulated by distinct signaling mechanisms; Journal Plant Physiology; 181:42-49; DOI 10.1016/j.jplph

Ildikó Vashegyi, Zsuzsa Marozsán-Tóth, Gábor Galiba, Petre I. Dobrev, Radomira Vankova, Balázs Tóth (2012) Cold Response of Dedifferentiated Barley Cells at the Gene Expression, Hormone Composition, and Freezing Tolerance Levels: Studies on Callus Cultures; Molecular Biotechnology, Part B of Applied Biochemistry and Biotechnology, ISSN 1073-6085, DOI 10.1007/s12033-012-9569-9

Krisztián Gierczik, András Székely, Mohamed Ahres, Zsuzsa Marozsán-Tóth, Ildikó Vashegyi, Wendy Harwood, Balázs Tóth, Gábor Galiba, Alexandra Soltész & Attila Vágújfalvi (2019) Overexpression of Two Upstream Phospholipid Signaling Genes Improves Cold Stress Response and Hypoxia Tolerance, but Leads to Developmental Abnormalities in Barley, Plant Molecular Biology Reporter, 314–326, DOI 10.1007/s11105-019-01154-5

Articles published in international journals related to other topics:

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