FINAL REPORT

ABSTRACT

In the present study we identified signal transduction components involved in cold acclimation and frost tolerance of barley and einkorn wheat using pharmacological approach. We investigated the possible utilization of callus cultures as experimental system to study frost tolerance and we observed that barley callus has altered cold response due to its different hormone content, therefore it is an unsuitable object for this kind of experiments. In barley seedlings we proved the role of calcium in the regeneration following freezing injury. In the gene expression level in barley we found that the cold induction of *CBF14* and *COR14b* appears to be dependent from the intracellular calcium release, while *CBF9* looks calcium independent and the whole system is regulated by phospholipase C. In einkorn wheat we observed that the cold induced expression of *CBF12* and *COR14b* was calcium dependent, while the expression of *CBF14* was not affected by the decrease of calcium response. Using microarray we found a series of calcium dependent cold responsive genes including the components of the antioxidant defense system. We investigated the effect of increased MAPK kinase activity on the cold response and we found the *CBF-COR* system independent from MAPK induction. We also investigated in an independent system the relationship of vegetative-generative transition and the expression of *COR14b* gene.

AIM OF THE STUDY

Our aim was to identify signal transduction pathways/components involved in the frost tolerance of cereals, namely in barley (*Hordeum vulgare*) and einkorn wheat (*Triticum monococcum*). For this purpose we planned to analyze the cold induced gene expression of the *CBF-COR14b* pathway in details using quantitative real-time PCR and its connection to the upstream pathways. This pathway is crucial in the cold acclimation and in the subsequent increased frost tolerance. Moreover, we planned to use microarray screen to identify cold induced genes in frost tolerant barley and we wanted to monitor how these genes depend from the upstream signaling events. Based on the data achieved in model organisms, our main target pathways were the phospholipase C and D (PLC and PLD) pathways. In experimental level a pharmacological approach was planned to modify our target signal transduction pathways in different crucial steps. We wanted to analyze the altered expression of cold induced genes after a pretreatment with different chemicals modifying the signaling pathway. The list of compound used can be seen in Table 1.

Table 1

CHEMICAL AGENT	PHARMACOLOGICAL EFFECT
EGTA	calcium chelator
lanthanium-chloride	calcium channel blocker
neomycin	PLC inhibitor
wortmannin	PI4K and PI3K inhibitor
thapsighargin	inhibitor of endoplasmic Ca-ATPase
ruthenium red	intracellular Ca-channel antagonist
mastoparan	MAPK inducer
U73122	PLC inhibitor
ionomycin	calcium ionophore
buthanol	PLD inhibitor

Initially, the whole study was designed to perform in callus culture based on the data describing similar frost tolerance and cold acclimation

existing in callus cultures compared to the whole plant. This appeared to be an ideal method since treating calli with chemicals is much easier than to work with hydroponics. Using callus cultures, the most challenging part of our study was to develop a fast, reliable system to monitor the frost tolerance of barley callus cultures following cold hardening.

RESULTS

Barley callus has altered cold response due to its different hormonal composition

From our main goals aimed at the beginning of the study, the development of a reliable callus system for frost tolerance determination was failed. Based on the data from the literature we developed a frost damage detection system using TTC and we confirmed and validated it by callus regeneration test. Although, we spent significant time of the project to set up the callus freezing system based on the data found in the literature, we were not able to reproduce those results. We initiated frost damage and increased the frost tolerance of calli as a result of cold hardening, but the rate of damage in the same temperature and using the same length of freezing was not reproducible. When we realized this, we decided to continue our experiments using young (7 days old) seedlings. In the same time, experiments were designed to find out the unreliability of the callus system in freezing tests. We observed that the consistence of the callus is very important in terms of homogeneity in the viability tests. The sucrose content of the original media should be increased to obtain 'greasy' calli, which were much useful for TTC detection. This modification was not enough for the increase of the reproducibility of our freezing system, so we had to look for other clues explaining our unexpected results. We investigated the cold induction of the *CBF-COR14b* system in barley seedlings and we found *CBF9*, *CBF14* and *COR14b* to be cold inducible. These results were in accordance with the data published previously. When we investigated the expression of these genes in callus, we found significant difference in the rate and the kinetics of the cold induction suggesting a different system existing in callus. Beside of our original goals we found it interesting to explain the different behavior of the *CBF-COR14b* system in callus. Since the dedifferentiation and maintenance of the calli requires high dosage of an auxin analogue called dicamba, we set up a rapid experiment to investigate the effect of this compound on the hormonal balance of the calli and we wanted to answer the question whether dicamba has any role in the cold induction of the *CBF9*, *CBF14* and *COR14b*. In collaboration with Dr. Radomira Vankova (Institute of Experimental Botany AS CR, Prague, Chech Republic) we investigated the hormonal content of cold treated or untreated calli grown on dicamba containing or dicamba deficient media and we found that the dicamba and the cold significantly changed the hormonal composition of the calli. Although these experiments were not part of the original project, we decided to report here and publish the results from this work in an international peer review journal during this year due to their novelty.

Frost tolerance of barley seedlings is calcium dependent

As a consequence of our findings with callus previously described, we decided to use young seedlings in hydroponics for our experiments. Since the traditional cold acclimation and freezing test protocol takes about 8 weeks, this long procedure does not allow the use of the inhibitors of signaling pathways due to their toxic effect during such a long term treatment. Consequently, we developed a rapid cold acclimation protocol, which is short enough to use the inhibitors, but long enough to result in significant cold hardening. Of course, using rapid hardening, the maximum level of frost tolerance can not be achieved, but our aim was to detect significant and reproducible increase of frost tolerance following as short time of cold acclimation as possible. In these experiments we determined that 7 days of cold acclimation

results in significant increase of frost tolerance. Based on the freezing tests we concluded that in the case of frost tolerant barley genotype 'Nure', the LT_{50} temperature during 1 hour freezing is -6°C using wet filter paper for the initiation of ice nucleation. These parameters were used in the subsequent experiments with the chemical compounds. Further preliminary experiments were performed to determine the lowest effective concentration of our inhibitors in the rapid freezing system and we found that only lanthanium (a calcium channel inhibitor) and EGTA (a calcium chelator) can be used for such a long experiment without significant damage of the seedlings. The other inhibitors were toxic in the effective concentration range. Based on the preliminary experiments a final protocol was established for freezing test using inhibitors. Briefly, 7 days old seedlings were pretreated with lanthanium (1mM) or EGTA (10mM) for 24 hours which was followed by one week of cold hardening (3°C/3°C; 16h/8h day/night regime). The plantlets were placed between two layers of wet filter papers and were frozen at different temperatures -6°C) for 1 hour using a liquid freezer. Frost damage was determined in a regeneration test by sowing the plantlets into soil, cutting back their leaves, keeping them under normal growth conditions (20°C/15°C; 16h/8h day/night) and monitoring the shoot length growth every third day. Comparing the treated and untreated plants we found that the inhibition of calcium response by blocking calcium channels using lanthanium or by chelating calcium using EGTA caused significant block of the regeneration following freezing suggesting the role of calcium in the cold acclimation and in freezing tolerance. We monitored inhibitor treated plants grown in normal condition and no change was observed in their regeneration rate excluding the non-specific effect of the calcium decrease. The results of this test are shown in Figure 1.

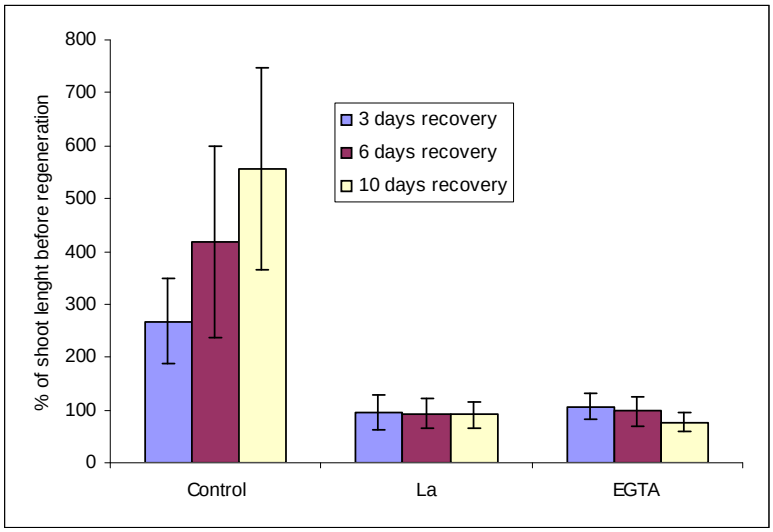

Figure 1. Frost test survival of seedlings pretreated with lanthanum or EGTA.

CBF-COR system is calcium dependent and it is regulated by the PLC pathway in barley

In the next step we investigated the effect of the modified calcium response, the modified PLC and PLD pathway on the *CBF-COR14b* expression system in barley. The chemical compounds used are shown in Table 1. The expression of *CBF* and *COR14b* genes following cold treatment was determined in different time points using quantitative real-time PCR. In control plants *CBF9*, *CBF14* and *COR14b* were found to be cold inducible. The expression of these three genes was analyzed after inhibitor pretreatment as well. During these experiments 24 hours pretreatment with different inhibitors was followed by 48 hours cold treatment

(3°C/3°C; 16h/8h day/night regime). Samples were taken for RNA extraction from the crowns of the seedlings at different time points.
First, we investigated the effect of our chemical agents on the expression of *CBF* and *COR* genes in plants grown in room temperature at different time points and we found that ionomycin, wortmaninn, thapsigargin, U73122 and buthanol reduce the gene expression even without cold treatment. Thus, these compounds were not used in experiments determining altered cold induction following pharmacological modification of signaling pathway.
Based on our frost test results, we investigated the effect of blocking the calcium response by lanthanium or EGTA on the cold induced expression of *CBF9*, *CBF14* and *COR14b* and we found that the calcium has major role on the induction of *COR14b*, but surprisingly the calcium depletion did not act negatively on the induction of *CBF9* and *CBF14* (Figure 2.).

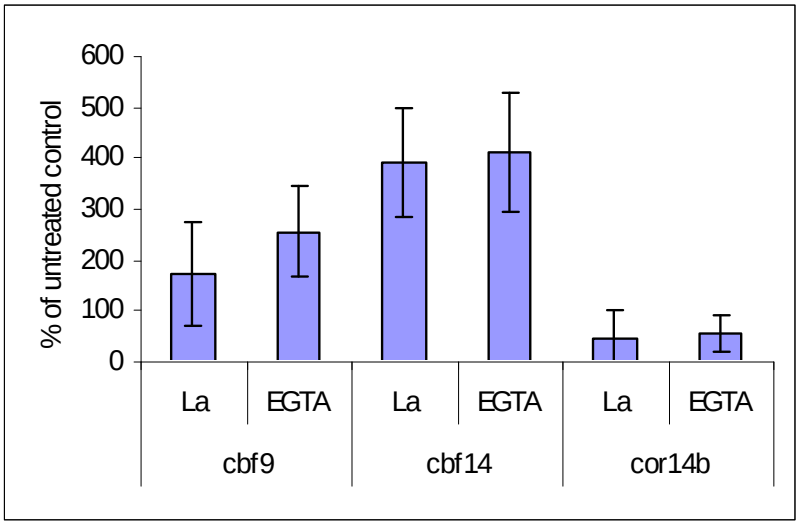

Figure 2. Expression level of cold-induced *CBF9*, *CBF14* and *COR14b* in barley following pretreatment with the calcium channel blocker lanthanum or the calcium chelator EGTA, determined by real-time PCR

As calcium appears to be crucial in the cold acclimation and subsequent increase in the frost tolerance, we analyzed and refined the calcium response involved. Using intracellular membrane Ca-channel antagonist, ruthenium red, we investigated the origin of the cold related calcium response and we found that the block of the intracellular calcium release significantly decreased the cold induced expression of *CBF14* and *COR14b*, while *CBF9* induction was not inhibited. This result suggests the existence of at least two distinct *CBF* related pathways in barley, *CBF14* and *COR14b* appears to be dependent from the intracellular calcium release, while *CBF9* looks calcium independent. We analyzed the role of upstream steps affecting calcium response using phospholipase C inhibitor neomycin and we observed the inhibition of the cold induced expression of *CBF9*, *CBF14* and *COR14b* suggesting their dependence from the PLC pathway.

CBF-COR system is calcium dependent in einkorn wheat

The calcium dependence of this process was confirmed in einkorn wheat as well. We found *CBF12*, *CBF14* and *COR14b* to be cold inducible in the frost tolerant genotype G3116, and using similar approach with lanthanium and EGTA we observed that the cold induced

expression of *CBF12* and *COR14b* was calcium dependent, while the expression of *CBF14* was not affected by the decrease of calcium response (Figure 3.).

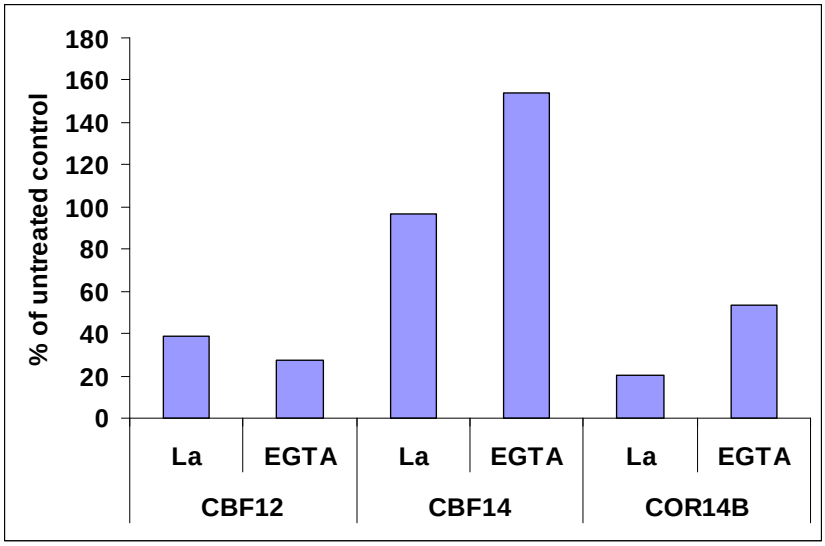

Figure 3. Expression level of cold-induced *CBF12*, *CBF14* and *COR14b* in einkorn wheat following pretreatment with the calcium channel blocker lanthanum or the calcium chelator EGTA, determined by real-time PCR

Calcium is important in the cold response of crucial regulating systems

As the calcium was shown to be essential for cold induced expression of *CBF-COR* system, we performed screening analysis in barley using Agilent 44K barley microarray to determine the calcium dependence of other cold responsive genes. In this screen we found more than 250 genes to be significantly cold induced and 90 of them showed calcium dependent cold response. Among these genes, components of the antioxidant defensive system (peroxidase, gluthathione S-transferase, MDHA reductase) were represented, moreover the genes of the previously analyzed *CBF-COR* mechanism and, obviously, calcium/calmodulin dependent protein kinases were found as well.

Mastoparan treatment affects several cold induced genes but not the CBF-COR system

Since the involvement of the MAPK kinase pathway in the frost tolerance is known in model organisms, we investigated the effect of the activation of this cascade on the induction of cold responsive *CBF* and *COR* genes in barley. The experimental system was set up similarly to the inhibitor pretreatment studies, but a known MAPK inducer, mastoparan was used in pretreatment, which was followed by cold induction and the expression of *CBF9*, *CBF14* and *COR14b* was investigated using real-time PCR. We concluded that neither the basal expression level nor the cold induced expression level of these three genes was altered by the mastoparan pretreatment. In a parallel experiment the effect of MAPK induction on cold responsive genes was investigated using Agilent 44K barley microarray using the same conditions as described before and we found about 140 cold induced genes to be down-regulated, while 53 genes showed increased expression. Due to the poor annotation of this

chip it is difficult to identify all the sequences, but it looks clear that the mastoparan treatment was effective and it did not acted on the *CBF-COR* system.

Additional results

It is known that freezing tolerance increases during acclimation of cereals in the vegetative stage, but decreases after the transition from vegetative to reproductive apices. In an independent system (*Triticum aestivum*) we investigated the negative effect of vernalization on the expression of *COR14b*.

CONCLUSION

The involvement of phospholipase C pathway and calcium in the frost tolerance has already been described in model organisms, but their role in the cold response of cereals was barely studied. In our project we provide novel evidences about the involvement of these signaling events in the cold acclimation and freezing tolerance in cereals. For the more accurate investigation of these mechanisms, a very reliable cell culture system would be ideal but the 2 years long duration of the present project did not allow us to establish such a methodological improvement.
The utilization of callus system for the study of cold response was also investigated and we concluded the unsuitability of it, due to its different hormonal composition. These experiments revealed the different cold responsive regulatory mechanism existing in callus compared to the whole plant.

JUSTIFICATION OF DEVIATIONS

Deviation in the list of participants

Ildikó Vashegyi as my PhD student was named as participating student in the original project proposal. In the first period of the project she was replaced to my other PhD student, Zsuzsa Tóth. Ildikó was involved in several other projects of the Department, but she played significant role in the completion of the present research grant, although she was not on the cost of the project.
Imrene Horvath was employed in the last months of the project as technical assistant, since we needed to compensate our delay.

Deviation from the research plan

As it is described in the report, the development of a reliable callus freezing system was failed. Since the duration of the project did not allow us to spend more time for the method development, we shifted our research object to the seedlings as an emergency solution,

because we had more background knowledge about them than we had about the callus system. Although, the callus freezing was failed, but we produced novel data about the different cold induced gene expression existing in callus and the effect of the hormonal content of calli on the cold stress response.
In the original research plan we wanted to confirm our barley microarray data in einkorn wheat, but we decided to put more effort on the more detailed study of *CBF-COR* system by real-time PCR, since very promising data were revealed from this system.

PUBLICATIONS

Our additional results is accepted for publication in Plant Biology
(A. Soltesz, I. Tımar, I. Vashegyi, B. Toth, T. Kellos, G. Szalai, A. Vagujfalvi, G. Kocsy & G. Galiba, Redox changes during cold acclimation affect freezing tolerance but not the vegetative reproductive transition of the shoot apex in wheat. Plant Biology. Accepted for publication; doi:10.1111/j.1438-8677.2010.00429.x)

Our preliminary results were presented as a poster in the meeting of „Stress responses - molecules, organisms and environments" organized by The Biochemical Society, The Society for Experimental Biology and the The British Ecological Society in London, in January 2011. (www.jointstress.org)
(Ildikó Vashegyi, Zsuzsa Tóth, Gábor Galiba, Balázs Tóth. Calcium dependence of COR14b related cold acclimation)

An oral presentation was held in the meeting of "Climate change - challenge for training of applied plant scientists"a Final Conference of the European Union Founded Project AGRISAFE in cooperation with EUCARPIA in Budapest, in March 2011. (http://www.agrisafe.eu/)
(Balázs Tóth, I. Vashegyi, Z. Tóth, E. Sebestyén, V. Soós and G. Galiba,. Calcium dependence of cold-regulated genes)

The results obtained from the investigation the effect of hormonal content of calli on the cold response, a publication is in progress in the Journal of Plant Growth and Regulation.

From the gene expression studies investigating the effect of PLC pathway and calcium on the frost tolerance and cold responsive genes, we plan a publication in an international peer review journal.