Final report of the project K104963 entitled "Role of light in the development of stress tolerance in cereals"

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Previous results clearly indicate that light play an important role in stress-related signalling during the adaptation processes. The main aim of this work was to obtain a better understanding of the responses of plants to certain stress factors under various light conditions. The project contains four main tasks:

Task 1. Characterization of the light-dependent role of particular genes in the development of cold hardiness

Task 2. Determination of the importance of light quality during the cold hardening period

Task 3. Role of light in the acclimation to various types of stressors in cereals

Task 4. Role of light in the cold acclimation mechanisms in the chilling sensitive maize

Results:

Task 1. Characterization of the light-dependent role of particular genes in the development of cold hardiness

In the present part of the work the expression level of selected genes from our previous microarray experiment (for detailed gene list see Majláth et al., 2012, Physiologia Plantarum 145: 296-314, suppl. material) will be tested in cereals with different levels of freezing tolerance to determine whether they show correlation with the freezing tolerance.

The experiments, which were carried out on durum wheat, were addressed to find the effect of the light intensity and water deprivation on the key steps of the carbon and nitrogen assimilation during cold hardening. The rate of carbon and nitrogen assimilation is highly sensitive to stress factors, such as low temperature and drought. Little is known about the role of light in the simultaneous effect of cold and drought. The present study thus focused on the combined effect of mild water deficiency and different light intensities during the early cold hardening in durum wheat (*Triticum turgidum* ssp. durum L.) cultivars with different levels of cold sensitivity.

Two durum wheat (*Triticum durum* L.) varieties with different frost tolerance (Mv-Makaróni frost tolerant and MvTD10-10 frost sensitive) were used for the experiments. During cold haredening (12 days at 4 °C) two different light intensities (normal light: 250 μmol m-2 s-1 (NL) and low light: 20 μmól m-2 s-1 (LL)) were used and a mild drought stress (D) was applied on part of the plants.

First the frost tolerance of the varieties was characterised. The frost test was carried out at -13 and -15 °C in complete darkness. The survival rate of drought-stressed plants was higher than the control's one. The survival of both varieties were similar at -13 °C at low light while at normal light the number of survivors was lower in the case of frost sensitive durum. At -15 °C the survival rate of the frost tolerant variety was higher hardened at both lights.

The results showed that reduced illumination decreased the undesirable effects of photoinhibition in the case of net photosynthesis and nitrate reduction, which may help to sustain these processes at low temperature. Mild water deficiency also had a slight positive effect on the effective quantum efficiency of PSII and the nitrate reductase activity in the cold. Glutamine synthesis was affected by light rather than by water deprivation during cold stress. The invertase activity increased to a greater extent by water deprivation, but an increase in illumination also had a facilitating effect on this enzyme. This suggests that both moderate water deficiency and light have an influence on nitrogen metabolism and sucrose degradation during cold hardening. A possible rise in the soluble sugar content caused by the invertase may compensate for the decline in photosynthetic carbon assimilation indicated by the decrease in net photosynthesis. The changes in the osmotic potential can be also correlated to the enhanced level of invertase activity. Both of them were regulated by light at normal water supply, but not at water deprivation in the cold. However, changes in the metabolic enzyme activities and osmotic adjustment could not be directly contributed to the different levels of cold tolerance of the cultivars in the early acclimation period.

In the second part of the work the sustaining effect of moderate drought on the quantum yield was confirmed at different illumination levels at low temperature in two durum wheat cultivars with contrasting cold tolerance. The kinetics of effective PSII quantum yield denoted differences in light utilization between the cultivars at low temperature. The cold-tolerant durum wheat cv. Mv Makaróni responded rapidly to the effect of moderate drought at both illumination levels and maintained a higher effective quantum yield at low temperature. The regulated way of fluorescence quenching was found to be responsible for the dissipation of excess excitation energy. The membrane damage was also reduced by moderate drought at low temperature in both cultivars as compared to irrigated plants.

Besides the acclimation effect of moderate drought, low light itself also helped to retain the quantum yield, maintain the photosynthetic pigment level and induce glycine betaine accumulation during the cold phase. A rise in the level of the osmoprotectant glycine betaine may contribute to the maintenance of the effective quantum yield. The present results provide new data for research on simultaneous abiotic stress effects in cereals.

A mansucript entitled „**The influence of moderate drought on glycine betaine helps to sustain the quantum yield of durum wheat in a light-dependent manner at low temperature**” is under preparation from the second part of the work and will be submitted soon. [1]

Task 2. Determination of the importance of light quality during the cold hardening period

In this part of the experiments the effect of the mutation on the light-dependence of the developing freezing tolerance during the cold hardening period was determined.

<u>The following mutants and transgenic lines were used in the experiments:</u>

Col-0 mutants:

[1] Only the manuscripts under preparation are indicated in this part of report. The list of already published papers can be found in the online part of the final report.

npq4: which due to a mutation gene encoding the PsbS protein, lacks the ΔpH and zeaxanthin-dependent conformational change in the thylakoid membrane that is necessary for non-photochemical quenching.

pif4: a mutant in the phytochrome-interacting bHLH factor functioning as a negative regulator of phytochrome B signalling.

Ler mutants and transgenic lines:

eid1-1: a new recessive mutant with increased sensitivity to far-red light (hypersensitive in phytochrom A-dependent high-irradiance responses).

YHB1: a phyB-5 null mutant of *Arabidopsis thaliana* Ler carrying a genomic fragment of AtYHB.

YHB2: a phyAphyB double null mutant of Arabidopsis thaliana Ler carrying a genomic fragment of AtYHB.

YHB3: a YHB transgenic line carrying the allele from the wild-type Arabidopsis thaliana Ler.

Conditions of plant growth, cold hardening and freezing

Arabidopsis seeds were planted into sterilized peat plugs (Jiffy products international AS, Norway) and stratified for 4 days at 4 ºC in dark. After that peat plugs were transferred into a Conviron PGR-15 growth chamber and plants were grown at 22/21 ºC, 10/14 h photoperiod with a light intensity of approximately 77 µmol m−2 s−1 and 75% relative humidity. 5 weeks later the first half of the plants were left at control temperature, but the second half of the plants was transferred into an other growth chamber where the environmental conditions were the same, except the temperature which was only 4 ºC. 5 days later leaf samples of a couple of control and cold-hardened plants of all genotypes were collected. The remaining plants were used for freezing test. A part of these plants were exposed to -8 ºC, the others were exposed to -10 ºC for 1 day in dark chambers. After freezing plants were transferred back to room temperature.

Results:

4 days after freezing (DAF) 100% of the plants which were exposed to -8 ºC before were stayed alive. However, after freezing at -10 ºC only Col-0 and npq4-1 survived in 100%, while pif4 and YHB3 survived in 50%, but survivals of the other genotypes were not found.

Gene expression analysis

There were no significant differences between the ABA biosynthesis genes expression levels of control plants of the investigated genotypes. At the leaf harvesting time the expression levels of these genes did not show unequivocal changes in the cold-hardened samples, except AtABA3 and AtAAO3. In the case of AtABA3 Col-0, pif4 and npq4-1 showed enhanced expression levels, while changes were not found in Ler, eid1-1, YHB1, YHB2 and YHB3. In the case of AtAAO3 only Col-0, YHB1 and YHB3 showed increased expression, in the control samples of these genotypes AtAAO3 was not expressed, but in YHB2 this gene was expressed both in control and cold-hardened samples.

Cold-hardening was not affected the expression level of AtCBF2, but it influenced AtCBF1 and AtCBF3. The expression level of AtCBF1 gene increased in YHB1, YHB3, Col-0 and pif4, but did not changed in the other genotypes, although in YHB2 it expressed both in control and cold-hardened samples of YHB2. AtCBF3 gene was activated by cold-hardening in Ler, YHB1, YHB2 and eid1-1, inactivated in YHB3, while it expressed with a same rate in Col-0, pif4 and npq4-1 after the 5 day long hardening.

Task 3. Role of light in the acclimation to various types of stressors in cereals

In the first set of experiments wheat plants were pre-treated with low concentration of salt (25 mM) and exposed to high level of salinity (500 mM) under different light conditions (300 μmol m^{-2} s^{-1} NL1 and 90 μmol m^{-2} s^{-1} LL1). High salinity under NL1 conditions caused a substantial decrease in the chlorophyll content in the non-acclimated plant; while those which were exposed to low concentration of salt only showed much less pronounced changes.

Exposure to high salinity under LL1 conditions also caused much less pronounced chlorotic symptoms then in plants treated under NL1 conditions. Chl a/b was also higher in plants pre-treated with low cc. of NaCl. These results indicate that salt acclimation may reduce the stress effects of high salinity; and light plays an important role in the development of damaging symptoms. Salt acclimation also led to a slight, but statistically significant higher level of carotenoids both with and without salt stress.

In order to characterise the physiological state of plants under salt acclimated conditions, gas exchange and chlorophyll-a fluorescence induction parameters were determined. Salt acclimation for 11 days with 25 mM NaCl led to a slight, but statistically significant decrease in the net assimilation rate, which is mainly due to the reduced stomatal conductivity.

In the next part of the work the following experiment was done: wheat plants were pre-treated with low concentration of salt (25 mM) and exposed to high level of salinity (500 mM) under different light conditions (300 μmol m^{-2} s^{-1} NL1 and 90 μmol m^{-2} s^{-1} LL1). After exposure to a high concentration of NaCl there was no difference in leaf Na content between the salt-acclimated and non-acclimated plants, indicating that salt acclimation did not significantly modify Na transport to the shoots. While the polyamine level was lower in salt-treated plants than in the control, salt acclimation led to increased osmotic potential in the leaves. Similarly, the activities of certain antioxidant enzymes, namely glutathione reductase, catalase and ascorbate peroxidase, were significantly higher in salt-acclimated plants. The results also suggest that while SOS1, SOS2 or NHX2 do not play a decisive role in the salt acclimation processes in young wheat plants; another stress-related gene, WALI6, may contribute to the success of the salt acclimation processes. The present work suggested that the responses of wheat plants to acclimation with low level of salt and to treatment with high doses of salt may be fundamentally different.

A microarray analysis was also planned for the third year using various stresses and various light conditions. The results of Task 4 (maize, hardening, chilling, see below) were more attractive so this analysis was carried out from this experiment. Detailed description in Task 4.

Task 4. Role of light in the cold acclimation mechanisms in the chilling sensitive maize

In order to characterise the role of light during the cold acclimation period in maize plants, two set of experiments were designed. One with relatively low growth and hardening light intensities, and a 2nd one with relatively higher light conditions, where the possible role of photoinhibition is more dominant.

In the **1st set** of experiments maize plants (*Zea mays* L. hibrid Norma) were grown in a modified Hoagland solution (Pál *et al.,* 2005) for a week at 22/20 °C at a photosynthetic photon flux density (PPFD) of 180 μmol m^{-2} s^{-1} (growth light 1; GL1) with 16/8 h light/dark periodicity. The plants were then hardened at 15/13 °C for 3 days under three different light conditions: GL1; low light intensity 1 (LL1): 46 μmol m^{-2} s^{-1}; and low light intensity2 (LL2): 14 μmol m^{-2} s^{-1}). After this the plants were transferred to 5 °C at continuous GL1 for three days, and then back to 22/20 °C for a 1-day recovery period. Leaf and root samples were collected for the estimation of viability and oxidative stress, and for carbohydrate analysis.

Results of the 1st set of experiments

The MDA level was measured to detect the extent of oxidative stress and was found to increase during hardening at GL1 in both leaves and roots, but did not change at LL1 or LL2. During chilling at GL1 the amount of MDA did not change significantly in the leaves but substantially increased in the roots. In the leaves of plants which were acclimated at low light intensity the chilling-induced increase in the MDA level was more pronounced. Interestingly, the light intensity during the hardening period also affected the chilling-induced MDA levels in the roots, where the lower the PPFD, the lower the MDA level detected.

The effect of light during the hardening period was also monitored by estimating the chlorophyll content. The SPAD values showed that although hardening in the light provided protection against subsequent cold-induced oxidative stress in the leaves, the chlorophyll content was slightly higher in plants hardened at lower light intensity.

These results suggest that although light may reduce the chilling-induced oxidative stress in maize, it may also have negative impacts on certain physiological processes probably due to its photoinhibitory effect, so the evaluation of the effect of light during cold acclimation in maize was continued by measuring electrolyte leakage. Cold acclimation at GL1 slightly increased the electrolyte leakage from leaf cells. However, after exposure to severe stress at 5 °C this increase was more pronounced in plants acclimated at LL1 and LL2.

The MDA data suggested that not only the leaves, but also the roots exhibited light-dependent cold responses. Since sugars are involved in several stress acclimation processes and their synthesis is directly dependent on light, the carbohydrate contents (glucose, fructose, sucrose, maltose) were investigated in the next step.

The fructose content rose slightly during hardening at GL1 in the leaves and roots, but significantly decreased in plants hardened at lower light intensities. During chilling stress a substantial increase was detectable in the leaves at all the light intensities tested. However, a similar increase could only be seen in the roots at GL1 and to smaller extent at LL2. Similar changes were observed in the glucose level, with the exception of the roots at LL1 and LL2, when the glucose level was higher than under control conditions. The sucrose content in the leaves decreased during hardening, but slightly increased at GL1 during chilling and to a great extent at LL1 and LL2. In the roots sucrose could only be detected during chilling and only in plants hardened at GL1 in relatively high amount. Similar changes were found in the case of maltose in the roots, while in the leaves the maltose profile was similar to that of glucose.

2nd set of experiments

In the **2nd set** of experiments maize plants were grown for 11 days at 22/20 °C at higher PPFD, (growth light 2, GL2 = 387 µmol m^{-2} s^{-1}) with 16/8 h light/dark periodicity. Some of the plants were then cold acclimated at 15/13 °C for 3 days either at GL2 or at moderately low light intensity 3 (LL3): 283 µmol m^{-2} s^{-1}; or at moderately low light intensity 4 (LL4): 107 µmol m^{-2} s^{-1}. Afterwards all the plants were transferred to 5 °C at continuous growth light (GL2) for 3 days, followed by a 7-day recovery period at 22 °C. Samples were collected for biochemical analysis from the control plants, and from treated plants after the acclimation, chilling and recovery periods.

Resulst

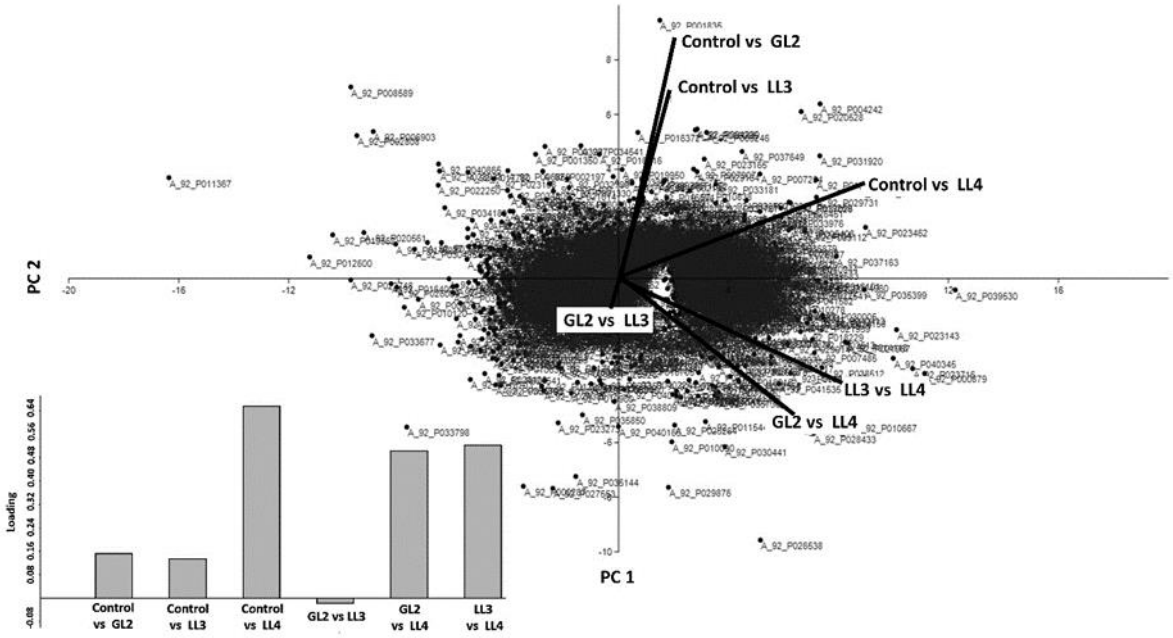
Since, despite its beneficial effects during the cold acclimation period, light may also induce secondary stress (photoinhibition), the 2nd set of hardening experiments was performed at higher light intensities. Since plants were generally adapted to this higher light during their growth period, neither cold acclimation nor chilling at 5 °C for 3 days caused substantial decrease in the chlorophyll content estimated with SPAD values (data not shown). However, visual analysis showed that the post-chilling symptoms during the recovery period (4 days at 22 °C after 3-day cold treatment at 5 °C), such as chlorosis in the distal parts of the leaves, were the most pronounced in un-acclimated plants, being less obvious in plants acclimated at higher light intensities.

The chlorophyll-a fluorescence induction technique provides a valuable tool to detect changes in Photosystem 2, especially under photoinhibitory conditions. Fv/Fm, which represents the maximum quantum efficiency of Photosystem 2, did not change significantly after growth at hardening temperature. However, it substantially decreased in a light-dependent manner at 5 °C in all the plants. Interestingly, this decrease was more pronounced in plants acclimated at higher light intensities. The decrease in Fv/Fm in non-acclimated plants exhibited values intermediate to those of plants acclimated at GL2 and LL. The hardening period this appears to have preconditioned the plants for the subsequent photoinhibitory effect of low temperature. The recovery of Fv/Fm was relatively fast. The quantum yield of Photosystem 2, Y(II), decreased within 3 days at 15 °C at GL2. However, at 5 °C Y(II) was not better in the non-hardened plants than in those hardened at GL2, and recovery was also slower than in acclimated plants. The negative impact of relatively high light intensity on the quantum yield was still detected during the beginning of the recovery period, but the decrease in this parameter was reversible within 7 days. The most significant changes in the regulated non-photochemical quenching, Y(NPQ), could be found in plants acclimated at GL2, where this parameter slightly increased; however, Y(NPQ) substantially decreased in the same plants after exposure to 5 °C for 3 days. This parameter recovered rapidly (within 1 day) at 22 °C. Non-regulated non-photochemical quenching, Y(NO), showed a similar pattern but with the opposite trend, substantially increasing in a light-dependent manner at 5 °C, especially in plants acclimated at GL2.

In contrast to the 1st set of experiments, where the light intensity was relatively low, the MDA content did not change substantially in the leaves in the 2nd set of experiments (Fig. 5). A notable increase could only be detected after 4 days of recovery, probably due to the post-chilling effect. However, as in the previous experiment, MDA showed a light dependent response in the roots, increasing even during the hardening period, especially at higher light intensity. Recovery was also light-dependent.

In order to obtain a better understanding of the molecular mechanisms underlying the role of light during the cold acclimation period in maize, gene expression analysis was performed using the microarray technique. Samples were taken from control plants growing at GL2 at 22 °C and

from plants hardened for 3 days at 15 °C at GL2, LL3 or LL4. In total, 42050 transcripts were examined on the chip. A scatter plot diagram and the loadings of the original variables were used to show the similarity between the global gene expression profiles of each comparison (Control vs GL2, Control vs LL3, Control vs LL4, GL2 vs LL3, GL2 vs LL4, LL3 vs LL4). The biplot representation showed that the Control vs GL2 and Control vs LL3 comparisons were similar to each other, but differed from GL2 vs LL3 in the expression profile along axis 1 (PC 1). The expression pattern of LL3 vs LL4 was similar to that of GL2 vs LL4 along axis 2 (PC 2). Control vs LL4 was quite dissimilar to the other comparisons. The results suggested that illumination levels GL2 and LL3 had a different effect on gene expression than the LL4 illumination level.



Scatter plot diagram of the expression datasets derived from principal component analysis based on the logFC values of the genes. All genes were plotted with respect to the first and second principal components (PC1, PC2). The axes represent PC1 and PC2, and the biplot representation shows the projection of the original variables (light regimes in the cold) onto the scatter diagram. Insert: The loadings of the original variables along PC1.

A pathway analysis is still in progress which can provide information about biochemical processes.

Some of the genes whose expression levels were significantly altered by the cold acclimating temperature and/or different light intensities during the hardening period, were involved in the carbohydrate metabolism.

The amounts of fructose, glucose, sucrose and maltose were determined in the leaves and roots after cold acclimation, after chilling treatment and during the recovery period. The highest fructose level was detected in the leaves of GL2 plants during hardening but dropped to the control level after three days at 5°C and remained at this level on the first day of recovery. Glucose and sucrose were detected in the highest amount in the leaves. As in the case of fructose, a great increase was detected in the leaves of GL2 plants during hardening, but hardly any change was observed after three days at 5°C and only a slight decrease during recovery. A similar change was detected in the amount of maltose during hardening, but its level increased at 5°C even in unhardened plants although the highest amount was measured in the leaves of

GL2 plants. During the recovery period it dropped back to the initial level. Fructose and sucrose increased in the roots in a light-dependent manner during hardening. The fructose content remained at the same level after three days at 5 °C, but the amount of sucrose increased, particularly in the case of GL2 and LL3. No substantial changes were determined in the levels of glucose and maltose in the roots.

In conclusion, the light intensity during the cold acclimation period may significantly affect cold acclimation processes in chilling-sensitive maize plants. In the present study it was shown that although photoinhibition may be dominant even at relatively high light intensity, light nevertheless contributes to the development of cold acclimation in maize. Due to the existence of photoinhibitory effects, the optimum light intensity depends on several factors. The light intensity during both hardening and growth periods may substantially affect the cold responses of maize. Interestingly, certain stress responses are light-dependent not only in the leaves, which sense the light directly, but also in the roots. As found earlier in frost-tolerant plants, light influences various light-related cold acclimation processes not directly, but at the gene expression and metabolomics levels. The results suggest that the photoinhibition induced by low temperature is a necessary evil for cold acclimation processes in plants.

The manuscript entitled "**Janus-faced nature of light in the cold acclimation processes of maize**" is under preparation from the above results and it is planned to submit in the near future.