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LIGHT-QUALITY-DEPENDENT MOLECULAR REGULATION OF FREEZING TOLERANCE IN BARLEY

Theses of the doctoral (Ph.D.) dissertation

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1. INTRODUCTION

The cultivation of cereals is very important to humanity, as they are not only a major part of our food, but in addition to animal feed, they also serve as a major source of raw materials for many industrial activities. Based on this, it is very important that our plants have a high yield safety. The main problem is the unpredictable weather conditions which are increasing year by year. As a result of unpredictable weather conditions yields can be much lower than the expected quantities. Changes in these weather conditions may lead to higher temperature fluctuations, which may prevent the development of frost tolerance in winter habit cereals. We face a similar problem in the spring, when overwintering plants lose their frost resistance and suffer cold damage as a result of subsequent frosts. The basis of our work is to study the protection against low temperatures and the early development of cold hardening.

Therefore, a fundamental question is how common the extreme temperature change or the daily average temperature fluctuation is in Hungary. Reviewing the data of the National Meteorological Institute of Hungary (https://www.met.hu/eghajlat/magyarorszag_eghajlata/eghajlati_adatsorok/Szombathely/data/monthly_data/) we can find that the average temperature in Szombathely was 9.37 °C in November during 1901-1930. The same parameter between 1981 and 2010 the temperature increased by almost 1 °C (10.01 °C). However, if we look at the daily temperature fluctuation, we are faced with the fact that the number of days below -4 ° C is increased by 6 days during the same periods. In other words, more extreme weather conditions actually occur in Hungary as well.

Based on all these facts, it can be seen that it is necessary to breed cereals that are much more resistant to these environmental factors. Although, to improve the acclimatization abilities of cereals can only be achieved by

understanding the major molecular mechanisms that play a role in abiotic stress responses in plants.

The most important part of the induction of cold hardening is the early detection of temperature drop. It has been described that the photoperiod and the light quality are important regulators in the cold acclimation processes through the modulation of the CBF regulon. During autumns, environmental changes (e.g. temperature, light intensity, spectra) are required for cereals to prepare physiologically to winter frost. It is also established that besides the cold the incident light also has a crucial role in the cold acclimation process. The most important environmental factors are the low temperatures, shortening of day length and alteration in the light spectrum, which are assumed to have a pivotal role in the induction of the cold hardening process. To elucidate the interaction between these external hardening factors two different experiments were carried out. For these experiments a cold-tolerant, winter habit barley *Hordeum vulgare spp. vulgare var.* Nure was used where we investigate the effects of the temperature, the light intensity and the light quality on the the development of frost tolerance.

2. OBJECTIVES

1. We want to elucidate the importance of the applied temperature, light intensity, and light quality in the process of cold acclimation. We want to investigate how light intensity, in parallel with the modified spectrum of incident white light, affects the freezing tolerance of the barley seedlings.

2. Study the expression of the *HvCBF14* gene and two well-characterized members of the CBF-regulon, the *HvCOR14b* and the *HvDHN5* genes, under modulated light and temperature conditions.

3. The elucidation of the mode of action of FR light (applied by artificial LED light sources) at moderate and low temperature on freezing tolerance, via modulation of phytohormone pools. The hormone levels and their metabolism were compared in winter barley under moderate temperature +/- FR, during cold stress after FR pre-hardening, and upon direct application of combined FR and cold treatment.

4. The investigation of the gene expression patterns of the genes involved in plant hormone biosynthesis.

3. MATERIALS AND METHODS

2.1. Plant Materials and Growth Conditions

For these series of experiments a cold-tolerant, winter habit barley *Hordeum vulgare spp. vulgare var*. Nure was used. In the first experiment after three days of germination, seedlings were planted in Jiffy-7 peat pellets (Jiffy Group, Oslo, Norway). In the second experiment seedlings were planted in wooden boxes (30 cm × 25 cm × 10 cm) filled with soil. The plants were grown in a PGV-36 growth chamber (Conviron PGV36; Controlled Environments Ltd.; Winnipeg, MB, Canada) equipped with a modular LED light ceiling. In the developing phase plants were cultivated for 14 d at a constant 15 °C temperature, with 12 h photoperiods under only white light with a continuous wide-spectrum LED (Philips Lumileds, LXZ25790-y) at 250 μmol $m^{-2} \cdot s^{-1}$ light intensity. Plants were irrigated three times a week with ½ Hoagland medium.

2.2. Light and Temperature Conditions during the First Experiment

We divided the ceiling into 6 areas, and in three areas the light intensities were 125, 250, and 350 μmol $m^{-2} \cdot s^{-1}$. We paired the rest of the areas, where we added supplementary FR illumination to the white light with a narrow 655 nm LED (Philips Lumileds, LXZ1-PA01). The red/far-red ratio was 0.5 above these plants. The temperature and the number of illuminated hours remained unchanged during the treatment, which lasted ten days. After 10 days the temperature was lowered to 5 °C without any other changes in the zones. This phase was seven days long. Samples were collected during the first and the last days of each treatment during a roughly 2-h period between 6 to 8 a.m. (ZT6 to ZT8) daytime.

2.3. Light and Temperature Conditions during the Second Experiment

The 18-d plants were separated into two areas. In one area white light at 250 μmol $m^{-2} \cdot s^{-1}$ intensity was supplied, and the plants in this zone served as control, whereas in the other zone white light was supplemented by FR illumination, with a narrow 750 nm LED (Edison Edixeon, 2ER101FX00000001), so that the R/FR ratio was modified to 0.5. The control zones did not contain any FR illumination. The whole experiment consisted of three different variants. Samples were collected during the first and the last days of each treatment during a roughly 2-h period between 6 to 8 a.m. (ZT6 to ZT8) daytime. Cold treatment was applied by gradually decreasing the temperature from 15 °C to 5 °C during the night, before the additional FR light was switched on in the morning.

The treatment variants from the second experiment:

- **FR-M:** The 18-day-old plants were exposed to FR at moderate temperature 15 °C for 10 days. During FR exposition, the plants reached the four-leaf stage.
- **FR-M/FR-C(28):** The 28-day-old (FR pre-hardened) plants from variant (1) were exposed to 5 °C for another 7 days (at high FR). At the end of the stress treatment the plants were 34 days old.
- **FR-C(18):** The 18-day-old plants were exposed directly to combination of cold 5 °C and high FR for 7 days.

2.4. Measurements of Electrolyte Leakage Levels in Leaf Samples

The leaf segments (2 mm long) from four different plants were placed in Falcon tubes (Thermo Fisher Scientific Inc., Wilmington, MA, USA). Subsequently, the samples were placed in a GP200-R4 liquid freezing system (Grant Instruments, Shepreth, UK) where a 50% ethylene glycol solution was flowing continuously to ensure rapid heat transfer and uniform temperature. The temperature was continually reduced from treatment temperatures to −2

°C, where we kept the samples for 18 h to simulate cold acclimation as a field circumstance. Samples from 15 °C temperature were frozen at −5, −7, and −9 °C, while the 5°C samples were frozen at −8, −10, and −12 °C for 1 h. After freezing, the samples were removed from the system, and MQ water was added to each tube. The samples were shaken for two hours before we measured the electrolyte leakage levels with a conductometer instrument (Mikro KKT, Budapest, Hungary). For the data analysis, Multi-Sample Conductometer version 1.0 (Intron Software, Biological Research Centre, Szeged, Hungary, (Copyright© L. Menczel, 2002)) was used. The relative conductance was calculated in five biological repetitions that originated from five different plants each.

2.5. Gene Expression (qPCR)

First total RNA was isolated using Direct-zolTM RNA MiniPrepkit (Zymo Research Corp., Irvine, CA, USA) and was determined by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, MA, USA). The cDNA libraries were prepared with the Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase and oligo (dT) 18 primer (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The expression level of genes was revealed by CFX96 TouchTM real-time PCR Detection System (Bio-Rad Hungary Ltd., Budapest, Hungary) and KAPA SYBR® FAST, Master Mix (2×), Universal qPCR Kit (Kapa Biosystems, Inc., Wilmington, MA, USA). The PCR primers were identical to previously published primers or we design them for the measurements. The relative expression levels were calculated by the ΔΔCt method. Cyclophilin was used as the reference gene.

2.6. Hormone Analysis

Leaf samples were homogenized with a ball mill (MM301, Retsch) and extracted in cold (−20 °C) methanol/water/formic acid (15/4/1 v/v/v). The following labelled internal standards (10 pmol/sample) were added: $^{13}C_6$-IAA, $^{2}H_2$-OxIAA (Cambridge Isotope Laboratories); $^{2}H_4$-SA (Sigma-Aldrich, St. Louis, MI, USA,); $^{2}H_3$-PA (phaseic acid), $^{2}H_3$-DPA (dihydrophaseic acid), $^{2}H_4$-7OH-ABA, $^{2}H_5$-ABA-GE (ABA-glucosyl ester) (NRC-PBI), $^{2}H_6$-ABA, $^{2}H_5$-JA, $^{2}H_5$-transZ, $^{2}H_5$-transZR, $^{2}H_5$-transZ7G, $^{2}H_5$-transZ9G, $^{2}H_5$-transZOG, $^{2}H_5$-transZROG, $^{2}H_5$-transZRMP, $^{2}H_3$-DHZ, $^{2}H_3$-DHZR, $^{2}H_3$-DZRMP, $^{2}H_7$-DZOG, $^{2}H_3$-DHZ9G, $^{2}H_7$-DZOG, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, $^{2}H_6$-iPRMP (Olchemim). Extracts were purified using a mixed mode reverse phase–cation exchange SPE column (Oasis-MCX, Waters). Two hormone fractions were sequentially eluted: (1) fraction A, eluted with methanol containing ABA, IAA, SA, and JA, and (2) fraction B, eluted with 0.35 M NH_4OH in 60% methanol containing CKs. Hormone metabolites were analyzed using HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems). Quantification of hormones was done using the isotope dilution method with multilevel calibration curves ($R2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems). Data are presented as means ± standard error. Three biological replicates were analyzed.

7.7. Statistical Analysis

For the statistical evaluation of the data, SPSS 16.0 was used. The homogeneity of the variances was checked by Levene's test, and normality was tested by Kolmogorov–Smirnov probe. To explore differences between treatments, a one-way ANOVA test was performed. Then, in justified cases

additional Dunnett T3 or Tukey's-b post hoc tests were applied. Student's t test was used to evaluate the results of the freezing test.

4. RESULTS

3.1. Results of the combined examination of temperature, light intensity and light quality (Experiment 1)

3.1.1. The Expression Patterns of *HvCBF14* and Its Target Gene *HvCOR14b*

FR supplementation at low light intensity (125 µmol $m^{-2}·s^{-1}$) caused a 5-fold increase in the HvCBF14 transcript level at 15 °C on the first day of the experiment, but after ten days this difference faded to 3-fold. After one day at a low 5 °C temperature, there was a large increase in HvCB14 gene expression (about 43-fold) even under white light illumination, but as a result of FR supplementation, this increase was even doubled. By the seventh day of the treatment, the expression levels had fallen, but the elevated pattern remained the same. At normal and high light intensity, 250 and 350 µmol m-2.s-1 respectively, similar gene expression patterns were obtained. The only important differences were the following: lower relative levels were achieved compared to the low-intensity results after cold exposure, but there were clear differences between the treated and untreated samples at all times.

For *HvCOR14b* the results were similar to *HvCBF14'*s patterns, so it confirmed its expression values as well. At low light (125 µmol $m^{-2}·s^{-1}$) and as a result of FR supplementation, *HvCOR14b* expression increased 11-fold on the first day, and this high level of expression was maintained until the end of the ten-day acclimation period at 15 °C. At low temperature, a staggering increase was measured. There was more than a 700-fold increase in the gene expression levels in the samples illuminated by white light, and still this value almost nearly doubled due to FR light supplementation after one day. However, the difference almost completely disappeared after the seven-day cold acclimation period. At normal (250 µmol $m^{-2}·s^{-1}$) light intensity there were also clear differences in all cases, similarly to the *HvCBF14* transcription

factor. At 15 ℃ the expression was increased 36-fold on the first day and 9-fold on the last day. At 5 ℃ a two-fold difference was measured between the light treatments in both cases, while the expression was significantly increased by the cold. At high light intensity, mRNA levels did not alter as a function of decreased R:FR ratio at 5 °C. There was no difference between the control and the FR treated samples; only the cold had an effect on the expression of this gene. However, at 15 °C FR supplementation caused a massive 93-fold increase in the transcript level after the ten-day treatment.

3.1.2. The Expression Levels of the HvDHN5 Gene

At low 125 PAR incident light intensity, the transcript level of the *HvDHN5* gene doubled as a result of FR supplementation on the first day at 15 °C, but this difference diminished after 10 days of the treatment. This tendency was also evident at 5 °C. Based on the results from samples treated at higher light intensities, it can be generally stated that neither the temperature nor the light treatments had a significant effect on the gene expression pattern. Of course, the gene expression levels were permanently higher in the samples originating from the lower temperature (5 °C).

3.1.3. Freezing test

In the case of plants grown at low (125 μmol $m^{-2} \cdot s^{-1}$) incident light intensity, at 15 °C a significant difference was observed, but only in samples that were frozen at −5 °C. The FR-treated plants were just around the LT50 (lethal temperature), while the control white light illuminated samples reached 75% lethality. In contrast, at 5 °C, which is low enough to induce cold acclimation, the plants exposed to modulated white light were more frost hardy. These plants reached the LT50 only after freezing at −12 °C, whereas in the case of the control, white light illuminated samples, the LT50 was reached at −8 °C. At increased light intensity (250 μmol $m^{-2} \cdot s^{-1}$), illumination

by white light with low R:FR ratio lowered frost-induced cell membrane injury in leaves even to a higher extent. When the highest light intensity (350 μmol $m^{-2} \cdot s^{-1}$) was applied, the relative conductance of the control samples decreased considerably. But, even in this case, FR supplementation caused significantly more reduction. Similar to the samples illuminated by lower light intensities, the freezing tolerance of the plants kept at low temperature (5 °C) were considerably higher than that of their counterparts grown at 15 °C.

3.2. Investigation in the hormonal composition of barley leaves at normal and low temperatures before and after far-red light supplementation.

3.2.1. The Effects of FR-Supplemented Light on Barley's Freezing Tolerance

In the first treatment at 15 °C (FR-M), the FR effect reached statistical significance only at a freezing temperature of −7 °C. The most remarkable freezing tolerance improvement between the W control and the FR-treated samples was observed at prolonged FR exposure FR-M/FR-C(28) (i.e., FR pre-hardening + low temperature treatment at high FR presence), both at -10 °C as well as at −12 °C. In the case of the combination of cold and light treatment (FR-C(18) and the corresponding W control), significant change occurred at a freezing temperature of −12 °C. Membrane damage was lower after plant acclimation to low temperature (5 °C) than in plants grown at 15 °C independently from light quality. In the case of the younger seedlings (18 days old at the beginning of the treatment) further improvement was observed both in the control W samples and in the FR supplementation, which further enhanced freezing tolerance. The results indicate that the FR enrichment positively influences the plant's freezing tolerance at low temperature. It may be concluded that freezing tolerance is affected by low temperature, FR enrichment in the case of W, as well as plant developmental stage.

3.2.2. Alterations in Plant Hormone Levels during Treatments

As phytohormones regulate cold acclimation in an intensive cross-talk, five hormones were followed in the experiments. The impact of FR was compared at different temperatures, treatment lengths, as well as plant ages. Significant differences were detected in the hormone levels and their metabolism, namely in case of ABA, JA, SA, IAA, and CKs, and especially of cis-zeatin-type.

FR treatment at 15 °C (FR-M) almost doubled the ABA content after 7 h, and that difference remained steady during the following 10 days. As expected, the cold treatment (5 °C) increased significantly ($p < 0.05$) the ABA content in the control W illuminated samples after 7 h, surpassing the level of the FR-treated samples. The temperature drop had only a moderate effect on the ABA concentration in the FR-acclimated samples after 7 h of cold. After 7 days of cold treatment, ABA content decreased both in the control W- and in the FR-treated samples (FR-M/FR-C(28)). At the beginning of the FR-C(18) treatment the plantlets were younger (18 days old) than at the FR-M/FR-C(28) (28 days) when the temperature dropped to 5 °C. Another difference was that in the case of FR-C(18) the experimental variant FR-treatment started together with the temperature shift. Both the W control and the FR-treated plants responded similarly to low temperature, although the ABA content in the FR-treated samples surpassed that of the W control samples after 7 h. Most likely the combination of the two external abiotic factors affected ABA metabolism synergistically. Interestingly, the elevated ABA content was partially preserved after a seven-day cold treatment both in the W- and FR-treated samples, a phenomenon that can be explained by the maintenance of an activated defense level in younger plants.

The FR supplementation had a moderate negative effect on JA levels at 15 °C (2-fold decrease after 10 days). The low temperature caused a transient down-regulation of JA content in all the samples. FR pre-treatment strengthened the cold effect in the FR-treated plants (4-fold decrease), especially at the early phase of the response. In the case of (FR-C(18)) treatment JA content was doubled within the first day of the cold stress, irrespective of light treatment, and increased still further after 7 days at 5 °C in W light.

Neither FR nor cold treatments affected SA content significantly during the first two treatments (FR-M, FR-M/FR-C(28)) and the corresponding W controls. During cold treatment of young plants (FR-C(18)), SA content changed similarly to JA. Upon cold exposure, SA content moderately increased in W, whereas this elevation was inhibited significantly ($p < 0.05$) by FR-supplementation.

IAA content was up-regulated almost two-fold during the first day of FR treatment at 15 °C, and that increase remained steady during the next 10 days (FR-M). The early response to cold was associated with IAA up-regulation in both the W- and FR-treated plants (FR-M/FR-C(28)). Nevertheless, in the last day of stress a moderate down-regulation was observed in the W illuminated samples. The FR treatment caused partial maintenance of IAA elevation. In the FR-C(18) experimental variant, combined stress caused a transient IAA down-regulation in FR-treated plants. After 7 days, the moderate IAA elevation may have indicated acclimation in the younger plants, irrespective of light treatment.

FR-treatment caused transient up-regulation in cZ content at 15 °C (FR-M) followed by its down-regulation. After the temperature decrease to 5 °C (FR-M/FR-C(28)), cZ content increased significantly in the W illuminated

samples. After FR pre-treatment, the supplementary FR light repressed this cold-induced elevation. In contrast, simultaneous application of cold and FR (FR-C(18)) enhanced cZ content 3-fold during 1 day compared to the W illuminated samples.

3.2.3. The Expression Patterns of the Key Hormone Metabolism-Related Genes

Transcription profiles of the ABA biosynthetic genes *ZEP1*, *NCED1*, *SDR2*, and *AO2* were determined. The expression of *ZEP1* varied only slightly during FR-M and FR-M/FR-C(28) treatments. In the FR-C (18) experiment, when the younger plants were cold treated, *ZEP1* transcript levels were elevated after 7 days independently from light quality. A large positive effect of FR light on *NCED1* expression was detected at 15 °C. At low temperature, *NCED1* expression was strongly down-regulated, independently from plant age or light spectra. The other two genes (*SDR2* and *AO2*) behaved similarly, and were clustered very closely together. Their expression was down-regulated, mainly by low temperature, except during the first day of the FR-M experiment, where the expression of *AO2* was slightly increased. The supplementary FR light, in synergy with the cold, further reduced transcript levels. In the FR-C(18) treatment this phenomenon was quite the opposite: instead of the decreasing pattern, an elevation was observed.

The genes related to JA and SA metabolism (*LOX* and *PAL*) belong to the same cluster, together with some ABA-related genes. The FR-treatment caused a mild decrease in the expression of these two genes after 10 days at 15 °C. An opposite pattern was observed in the FR-M/FR-C(28) and FR-C(18) experiments, the former one was associated with a mild down-regulation, whereas the latter one with up-regulation (especially of PAL).

The *COAA* and *YUCCA5* genes were investigated as important genes in auxin biosynthesis. The largest changes were observed in the case of *COAA*. A 3-fold stimulation of expression was caused by FR supplementation at 15 °C after 7 h (FR-M). Comparison of FR-M/FR-C(28) and the corresponding W control showed that low temperature eliminated the differences between light treatments. The cold treatment of 18-day-old plants resulted in even larger increases, independently from light quality.

The expression pattern of *CKX9* was clustered to *NCED1*. A large, six-fold elevation of *CKX9* expression was observed upon FR supplementation to W at 15 °C (FR-M). At low temperature, *CKX9* expression decreased dramatically in all varaints.

5. CONCLUSIONS

In the first experiment our aim was to study the expression of the HvCBF14 gene and two well-characterized members of the CBF-regulon the HvCOR14b and the HvDHN5 genes. These genes were observed under modulated light (white and FR supplemented white light) and temperature conditions (15 °C and 5°C). We also elucidated how light intensity, in parallel with the modified spectrum of incident white light, affects the freezing tolerance of the barley seedlings.

According to our best knowledge, these are the first results to show that the combined effects of cold, light intensity, and modification of the R/FR light ratio can greatly influence the expression pattern of HvCBF14 and HvCOR14b genes and can also adjust the level of the HvDHN5 gene. Interestingly, the two genes, which are used primarily as markers to predict the level of barley frost tolerance, behaved differently. While the HvCOR14b gene showed an absolute increase in both low-temperature and under supplemented far-red light conditions, HvDHN5 only responded to low temperatures in a reliable manner. This suggests that the HvDHN5 gene should be used when light modifications do not occur or to predict the level of total hardiness after adequately long cold acclimation. Therefore, we suggest that in those cases, when artificial light sources are used during cold hardening, the expression level of HvCOR14b should be monitored instead. These three environmental factors seem to be interfering with each other during the cold acclimation process, which can result in serious alterations in the level of frost hardiness. Accordingly, during selection for frost tolerance in plant growth chambers, it is very important to keep both the light intensity and spectrum constant, and this is the prerequisite for repeatability. Moreover, in field conditions applying external light sources, it also might prevent cold injuries in different plant species during autumn.

In the second experiment the effects of the FR supplementation on barley plants was further investigated but only at 250 $m^{-2} \cdot s^{-1}$ light intensity. In this experiment the impact of reduced R/FR ratio in the incident white light was studied on the hormone levels, and on the key hormone metabolism-related genes in winter barley leaves at moderate (15°C) and low (5°C) temperature.

We proved that the FR-supplemented white light acts as a coordinator in the pre-hardening process, aside from its regulation of many plant developmental and physiological processes. Supplementary FR light had a positive impact on the plants' cold tolerance, even at moderate temperature which also confirmed the results of the first experiment. This effect was strengthened by cold treatment at 5 °C, in comparison with a non-cold hardening temperature. FR-enhanced freezing tolerance at 15°C was associated with promotion of abscisic acid (ABA) levels, accompanied by moderate increase of indole-3-acetic acid (IAA) and cis-zeatin (cZ) levels. The most prominent impact on plants' freezing tolerance was found after FR pre-treatment at 15°C followed by cold treatment together with FR supplementation. Response of ABA was diminished in comparison with white light treatment, probably due to the previous transient elevation of ABA content during FR pre-treatment. Jasmonic acid (JA) and salicylic acid (SA) were transiently reduced. When the plants were exposed directly to a combination of cold (5°C) and FR supplementation, ABA increase was higher than in white light, and was associated with enhanced elevation of JA. After sevent days of the combined treatment IAA and cis-zeatin also increased, which indicate stronger stress response and better acclimation. Cold hardening was more efficient when FR light was applied in the early developmental stage of barley plants rather than in later stages. The results of the hormone and the qPCR analyses indicate that the temperature had a greater impact on the plant

behaviour than the light spectra modification. Both synergetic and antagonistic effects were found among these two environmental cues when applied together. The timing and duration of the FR treatment seem to be a crucial factor in the cold acclimation processes as far as plant acclimation is concerned. Another important factor is plant age. Cold hardening was more efficient in the early developmental stages of barley plants, which was associated with higher stimulation of plant defence as indicated by comparable elevation of ABA and higher increase of JA and SA.

This series of experiments may serve as an evidence for the close relationship between plant hormones, light qualityand low temperature in the beginning of cold acclimation. After serious alterations in phytohormone contents and in their related transcriptome by the decreased R/FR ratio and/or the low temperature, an elevated frost tolerance was measured in all instances. Based on these results it seems that an elevated protection against the low temperature can occur in plants as a positive side effect of the shade avoidance syndrome. However, it is important to emphasize the timing of the FR treatments are also a key factor for this positive influence. The better timing for the application of modulated light spectrum results in higher hardening state during the pre-hardening process.

6. NEW RESULTS (THESES)

1. According to our best knowledge these are the first results to show that the combined effects of the cold, light intensity and the modification of the R/FR light ratio can greatly influence the expression pattern of the HvCBF14 and the HvCOR14b gene, and also adjust the level of the HvDHN5 gene. While low temperature induced all of their expression, far-red light supplementation was able to further enhance their transcriptional level except for the HvDHN5 gene. This positive effect was sometimes suppressed or further strengthened by the change in light intensity.

2. The two genes which are used primarily as markers to predict the level of the barley frost tolerance behaved differently. While the HvCOR14b gene showed an absolute increase in both low temperature and under supplemented far-red light, the HvDHN5 only responded to the low temperature in a reliable manner. This suggests that the HvDHN5 gene should be used when light modifications do not occur or to predict the level of total hardiness after adequately long cold acclimation.

3. These three environmental factors seem to be interfering with each other during the cold acclimation process. The modification of temperature, light intensity and R/FR ratio have an additive effect on barley's cold hardiness, but in many cases they cause even synergistic effect in the barley plants. This phenomenon leads some serious alteration in the level of frost hardiness in the early stages of cold hardening.

4. Cold hardening was more efficient in the early developmental stages of barley plants, which was associated with higher stimulation of plant defence as indicated by comparable elevation of ABA and higher modulation of JA and SA.

5. The timing and duration of the FR treatment seem to be crucial factors in cold acclimation processes as far as plant acclimation is concerned.

6. Based on these results it seems that elevated protection against low temperature can occur in plants as an adaptation to autumn-related environmental changes, distinct from the shade avoidance syndrome.

7. The results of the hormone and the qPCR analyses indicate that temperature had a greater impact on plant behaviour than the modification of the light spectrum.

8. FR-supplemented white light acts as a coordinator in the pre-hardening process, apart from the regulation of many plant developmental and physiological processes.

PUBLICATIONS RELATED TO THE PRESENT STUDY

Reseach papers with impact factor:

Ahres, M.; Pálmai, T.; Gierczik, K.; Dobrev, P.; Vanková, R.; Galiba, G. The Impact of Far-Red Light Supplementation on Hormonal Responses to Cold Acclimation in Barley. Biomolecules 2021, 11, 450. https://doi.org/10.3390/biom11030450

Ahres, M., Gierczik, K., Boldizsár, Á., Vítámvás, P., & Galiba, G. (2020). Temperature and Light-Quality-Dependent Regulation of Freezing Tolerance in Barley. PLANTS-BASEL, 9(1), 83. http://doi.org/10.3390/plants9010083

Gyugos, M., **Ahres, M.,** Gulyás, Z. et al. Light spectrum modifies the drought-induced changes of glutathione and free amino acid levels in wheat. Acta Physiol Plant 43, 90 (2021). https://doi.org/10.1007/s11738-021-03253-x

Kovács, T., **Ahres, M.,** Pálmai, T., Kovács, L., Uemura, M., Crosatti, C., & Galiba, G. (2020). Decreased R:FR Ratio in Incident White Light Affects the Composition of Barley Leaf Lipidome and Freezing Tolerance in a Temperature-Dependent Manner. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES, 21(20). http://doi.org/10.3390/ijms21207557

Conference full papers and abstracts :

Ahres, M., Boldizsár, Á., Gierczik, K., Székely, A., Vágújfalvi, A., & Galiba, G. (2019). Az árpa fagyállóságának változása különböző fényspektrumok és hideghatás következtében. In Növénynemesítés a 21. század elején: kihívások és válaszok (pp. 204–208).

Ahres, M., H. Kamiran, Á., Gierczik, K., Boldizsár, Á., Vanková, R., & Galiba, G. (2019). Temperature dependent hormonal and metabolomics changes during supplementary far-red light induced pre-hardening process in barley. In 5th Conference on Cereal Biotechnology and Breeding Book of abstracts (pp. 40–40).

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