Thesis of doctoral (Ph.D.) dissertation

Redox control of metabolism in wheat

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Introduction

Wheat (*Triticum aestivum* L.) is the world's largest frequently grown cereal, with over 220 million ha cultivated annually under a wide range of climatic conditions [1]. Wheat grains contain 8-20% protein and trace amounts of antioxidant enzymes, minerals, and vitamins such as thiamine, riboflavin, niacin, and vitamin E [1,2]. Environmental stressors that caused oxidative stress induce various biochemical, molecular, and physiological changes in the plant's primary metabolism, leading to reduced yield. These effects, however, are mostly dependent on the severity and length of the stress conditions.

Among the inducers of oxidative stress, changes in light intensity or spectrum are very important, the alteration of which may result in pronounced modifications of wheat metabolism [3–7]. It is widely reported, that high light-induced oxidative stress modified the redox status of plants [8–13]. Similar to extreme light conditions, low temperature may also lead to redox imbalance and oxidative stress in wheat [14–16]. The C-repeat binding transcription factors (CBF) are important regulators of transcriptional and metabolic changes in cereals and other plant species during cold acclimation [17,18]. Like changes in light and temperature conditions, the excess of oxidants (for example hydrogen peroxide: H_2O_2) and reductants (for example ascorbate: ASA) could also disturb the redox homeostasis, thereby influencing the metabolism in plants. It was revealed that the exogenous application of H_2O_2 could induce oxidative stress in plants depending upon the concentration and duration of its application [19,20]. The application of a reductant (dithiothreitol) to *Arabidopsis* stimulated organic and amino acid synthesis while reducing sucrose synthesis [21]. Furthermore, foliar ASA treatment induced the deposition of free amino acids and soluble carbohydrates in flax cultivars [22]. The regulating effect of oxidants has been studied more extensively in plants; however, the potential deleterious effect of reductants applied in high concentrations or for a prolonged period has only been evaluated in a few studies and has not been clearly understood, yet.

Although the influence of the environmental factors on the redox system and metabolism was examined in several plant species, the effect of light intensity and spectrum on the glutathione metabolism under optimal and low temperature was not compared earlier in wheat genotypes with different freezing tolerance. In addition, an extensive comparative study about the effect of an oxidant and reductant on wheat metabolism was not carried out until now. The aim of this thesis was the investigation of the aforementioned scientific questions.

Aims

We aimed to investigate the modulating effect of light conditions on cold acclimation and the regulation of the metabolism by reducing and oxidising compounds in wheat. Therefore, this study was done to get answers to the following questions:

1) What is the role of light (intensity and spectrum) in the regulation of redox homeostasis in wheat seedlings?

2) How do the light-dependent redox changes affect the biochemical processes during the subsequent cold period in wheat seedlings?

3) How does the exogenous application of an oxidant (H_2O_2) and a reductant (ASA) modify the redox environment and the expression of the redox-responsive genes in wheat?

4) How do the changes in the redox environment modulate the primary metabolite profile in wheat?

Materials and methods

For the study of the effect of light conditions on the cold-induced physiological and biochemical changes two freezing-tolerant (Cheyenne – winter growth habit; Miranovskaya 808 – winter growth habit) and two freezing-sensitive (Cappelle Desprez – winter growth habit; Chinese Spring – spring growth habit) wheat (*Triticum aestivum* L.), genotypes were used in the current experiments [23,24]. 14-day-old seedlings were transferred to three light conditions: normal light intensity, high light intensity, and supplementary far-red light [25]. The subsequent cold treatment at 5 °C continued for a week and the recovery period lasted for 3 weeks at 20/17 °C [26].

The effect of ASA and H_2O_2 on primary metabolism and redox homeostasis was investigated in the wheat (*Triticum aestivum* L.) cultivar (Chinese Spring) involved in this experiment [27]. The 14-day-old seedlings were subjected to 0, 5, and 20 mM ASA (reductant) and H_2O_2 (oxidant). The sampling for physio-biochemical and molecular determinations was done following 0-, 3- and 7-day treatments.

The relative electrolyte leakage, chlorophyll (*a* and *b*) and carotenoid contents, H_2O_2 content, lipid peroxidation, and activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), catalase (CAT), and glutathione s-transferase (GST) were measured by an UV–visible spectrophotometer. The photosynthetic activity of the leaves was measured by the use of a Ciras 3 Portable Photosynthesis System. Ascorbate and dehydroascorbate, non-protein thiols were detected by high-performance liquid chromatography. The measurements of metabolites were carried out by gas chromatography-mass spectrometry. An automatic amino acid analyser was used for the determination of free amino acids.

Summary

The sessile organisms, plants experience various abiotic stresses during their life span such as light (intensity and spectrum), extreme temperatures (low or high), exogenous application of the chemicals/compounds, water deficit conditions, soil salinity or heavy metals which ultimately result in ROS production, and in turn in disturbance of the redox homeostasis. Therefore, besides the study of the effect of light and cold, we have

simulated the influence of the modified redox environment of the tissues by ASA and H_2O_2 treatments in order to see their effects on various physiological, biochemical, metabolic, and molecular mechanisms in wheat seedlings. For this purpose, two experimental systems were used:

During the study of the effect of light conditions on the response to cold treatment, two freezing-tolerant (Cheyenne – Ch, winter growth habit; Miranovskaya 808 – Mir, winter growth habit) and two freezing-sensitive (Cappelle Desprez – CD, winter growth habit; Chinese Spring – CS, spring growth habit) wheat (*Triticum aestivum* L.) genotypes were used. Three light treatments/conditions were as follows: white light; 250 µmol m^{-2} s^{-1} (denoted as normal light intensity; red/far-red ratio – 15:1; blue/red ratio – 1:2), white light; 500 µmol m^{-2} s^{-1} (denoted as high light intensity, blue/red ratio - 1:2; red/far-red ratio - 15:1) and far-red light; (blue/red ratio of 1:2, red/far-red ratio - 10:1) with 250 µmol m^{-2} s^{-1}. The subsequent cold treatment at 5 °C continued for a week and the recovery period lasted for 3 weeks at 20/17 °C. The sampling for physio-biochemical and molecular analysis (electrolyte leakage, enzyme activity, thiols, and gene expressions) was done before (14 days old plants) and after (21 days old plants) the cold treatment.

High light intensity and supplementary far-red light modified the redox environment through their influence on the level of the non-protein thiols and activities of ASA-GSH cycle-related enzymes in the shoot tissues of wheat. This led to an improved cold acclimation process as indicated by the increased freezing tolerance of leaf segments based on the reduced membrane damage. There was a positive correlation between freezing tolerance levels and the redox state of the GSH pool. The redox environment in the shoot tissues of freezing-sensitive genotypes became more oxidising compared to the tolerant ones under the modified light conditions, which can adversely affect the activities of the redox-sensitive proteins and the amount of the transcripts linked to the reduction of freezing-induced damage.

The influence of ASA and H_2O_2 on metabolism was studied in the wheat (*Triticum aestivum* L.) cultivar (Chinese Spring). The seedlings were treated with 0-, 5-, and 20-mM ASA (reductant) and H_2O_2 (oxidant). The sampling for physio-biochemical and molecular determinations (membrane injury, lipid peroxidation, hydrogen peroxide, photosynthetic parameters, enzyme activity, thiols, ascorbate and dehydro-ascorbate, metabolite profiling, amino acids, and gene expressions) was done following 0-, 3- and 7-day treatments in the middle of photoperiod.

Chemical modification of the redox state in the shoot tissues altered the metabolic processes. The longer (7 days) and higher concentrations (20 mM) of the reductant (ASA) and oxidant (H_2O_2) converted the redox environment of the wheat leaf tissues into a more oxidized direction and more reduced direction based on the size and redox status of the ascorbate and glutathione pools, respectively. These redox variations caused different modifications in the primary metabolic processes as shown by the free amino acid, carbohydrate, and organic acid levels. An adjustment exists, at least partly at

the transcriptional level, and may be involved in the recovery of the redox state existing under control conditions.

The most important results obtained in the two experimental systems:

1) High light intensity decreased the electrolyte leakage (an indicator of the membrane damage) at the end of the cold treatment only in the two tolerant genotypes, but not in the two sensitive ones. This observation indicates that high light intensity activates the cold acclimation processes in a cold tolerance-dependent manner. Far-red light did not exhibit such effect in the present experimental system.

2) Both high light intensity and supplementary far-red light greatly increased the formation of γ-glutmylcysteine (intermedier product of glutathione (GSH) synthesis) and cysteinylglycine (GSH degradation product) during cold in all genotypes independently of their freezing tolerance. This change resulted in a great reduction of the γ-glutamylcystine (γESSE)/γ-glutamylcysteine (γEC) and cystinylglycine (CySSGly)/cyteinylglycine (CysGly) ratios; therefore the pool of both thiols became more reduced.

3) The cold-induced increase in GSH content was not or only slightly affected by the light intensity or supplementary far-red light. However, the GSSG content and consequently the GSSG/GSH ratio was greatly reduced before the cold treatment in supplementary far-red light in the two frost-sensitive genotypes, but not in the two tolerant wheat genotypes. The supplementary far-red light greatly induced the hmGSH and hmGSSG accumulation during the cold treatment only in the two sensitive genotypes. These findings indicate the frost-tolerance-associated control of (hm)GSH metabolism in wheat.

4) At normal growth temperature, lower activities of the antioxidant enzymes were observed under high light intensity in all genotypes except for APX and DHAR in CS. A similar decrease only occurred under the other two light conditions following the cold treatment. These observations show the interactive effect of light and temperature on the antioxidant enzymes.

5) In contrast to the cold-induced decrease of the antioxidant enzyme activities in normal light and supplementary far-red light, the transcription of several of them even increased during cold treatment. Thus, their activities are not controlled at the transcriptional level.

6) ASA and H_2O_2 significantly reduced the studied photosynthetic parameters. The disturbed photosynthesis resulted in a similar increase in lipid peroxidation and electrolyte leakage (membrane damage) after both treatments.

7) Interestingly, the endogenous H_2O_2 level was greater after the exogenous application of ASA than following H_2O_2 treatment. Due to this difference, the redox state converted

into more oxidized following ASA application and more reduced following H_2O_2 supplementation based on the ratios of oxidised and reduced ascorbate and glutathione.

8) The excessive ASA could repress, whereas H_2O_2 could stimulate the OPPP and glycolysis pathways producing reducing power as indicated by the unaffected and declined glucose 6-phosphate content, respectively. This difference may explain the greater H_2O_2 content after ASA treatment.

9) The proposed inhibition of the glycolysis and subsequently the citrate cycle by ASA and their induction by H_2O_2 may be responsible for the smaller and greater synthesis of amino acids from the intermediers of these pathways, respectively.

10) Following ASA addition, the more oxidising environment stimulated several transcripts related to the ASA-GSH cycle and the pentose phosphate pathway, which can contribute to the subsequent recovery of the optimal redox state. In contrast, after H_2O_2 treatment this effect was much weaker or did not exist.

Összefoglalás

A növények helyhez kötött élőlényként életük során különféle abiotikus stressz hatásoknak vannak kitéve, mint például a megváltozott fényviszonyok (intenzitás és spektrum), szélsőséges hőmérséklet (alacsony vagy magas), a mezőgazdaságban használt vegyszerek, vízhiány, a talaj nagy sótartalma vagy nehézfémek. Ezek ROS felhalmozódáshoz vezetnek, mely a redox homeosztázis egyensúlyát megzavarva oxidatív stresszt idéz elő. A fény és a hideg kezelések következményeinek vizsgálata mellett a különböző kedvezőtlen környezeti tényezők hatásait a szövetek redox környezetére ASA-val és H_2O_2-val történő kezeléssel szimuláltuk. Ezen kezelések befolyását a különböző élettani, biokémiai, metabolikus és molekuláris folyamatokra fiatal búzanövényekben tanulmányoztuk, két kísérleti rendszert használva:

A fényviszonyok hidegakklimációra gyakorolt hatásának vizsgálata során két fagytűrő (Cheyenne – Ch, őszi; Miranovskaya 808 – Mir, őszi) és két fagyérzékeny (Cappelle Desprez – CD, tavaszi; Chinese Spring – CS, tavaszi) búza (*Triticum aestivum* L) genotípust hasonlítottunk össze. A három fénykezelés a következő volt: fehér fény; 250 µmol m^{-2} s^{-1} (normál fényintenzitás; vörös/távoli vörös arány – 15:1; kék/vörös arány – 1:2), fehér fény; 500 µmol m^{-2} s^{-1} (nagy fényintenzitás, kék/vörös arány - 1:2; vörös/távoli vörös arány - 15:1) és távoli vörös fény; (250 µmol m^{-2} s^{-1}; kék/vörös arány 1:2, vörös/távoli vörös arány - 10:1). Az ezt követő hidegkezelés 5 °C-on egy hétig, a regenerációs időszak pedig 3 hétig tartott 20/17 °C-on. A biokémiai és a molekuláris analízisre (elektrolit-kiáramlás és enzimaktivitás mérések, tiol tartalom meghatározása és génexpressziós elemzések) a mintavételezés a hidegkezelés előtt (14 napos növények) és után (21 napos növények) történt.

A nagy fényintenzitás és a kiegészítő távoli vörös fény a nem-fehérje tiolok szintjére és az ASA-GSH ciklushoz kapcsolódó enzimek aktivitására gyakorolt hatásuk révén módosították a redox környezetet a búza hajtásszöveteiben. Ez a hideg akklimációs folyamat javulásához vezetett, amit a levélsejtek megnövekedett membránstabilitása jelez a csökkent ionkiáramlás alapján. Pozitív korrelációt találtunk a fagytűrés szintje és az összes glutationtartalom között. A fagyérzékeny genotípusok hajtásszöveteiben a redox környezet a módosított fényviszonyok mellett a toleránsakhoz képest oxidálóbbá vált, ami hátrányosan befolyásolhatja a redox-érzékeny fehérjék aktivitását és a fagytűrőképességgel összefüggő transzkriptumok mennyiségét.

Az ASA és a H_2O_2 metabolitprofilra gyakorolt hatását a Chinese Spring búzafajtában (*Triticum aestivum* L.) vizsgáltuk. A fiatal növényeket 0, 5 és 20 mM ASA-val (redukálószer) és H_2O_2-vel (oxidálószer) kezeltük. A biokémiai és a molekuláris meghatározásokra (elektrolit-kiáramlás, lipidperoxidáció és hidrogén-peroxid tartalom meghatározása, fotoszintetikus paraméterek és enzimaktivitás mérése, oxidált és redukált tiol, aszkorbát és dehidroaszkorbát tartalom valamint metabolitprofil meghatározása, aminosav és génexpressziós elemzések) a mintavételezés 0, 3 és 7 nappal a kezelés után történt a fotoperiódus közepén.

A redox környezet kémiai módosítása a hajtásszövetekben megváltoztatta az anyagcsere folyamatokat. A redukálószeres (ASA) és az oxidálószeres (H_2O_2) kezelés hosszabb időtartama (7 nap) és nagyobb mértéke (20 mM) a búzalevél szöveteinek redox környezetét az aszkorbát és a glutation redox állapota alapján az oxidáltabb, illetve a redukáltabb irányba tolta el. Ezek a redox változások különböző módosulásokat okoztak az elsődleges anyagcsere-folyamatokban, amit a szabad aminosavak, a szénhidrátok és szerves savak szintjének változása jelez. A szabályozás legalább részben transzkripciós szinten zajlik, és szerepet játszhat a környezeti hatásokra megváltozott redox környezet helyreállításában.

A két kísérleti rendszerben elért legfontosabb eredmények:

1) A nagy fényintenzitás csak a két fagytűrő genotípusban csökkentette az elektrolitkiáramlást (membránkárosodás jelzője) a hidegkezelés végén, a két érzékeny genotípusban viszont nem. Ez a megfigyelés arra utal, hogy a nagy fényintenzitás fagytűrés-függő módon aktiválja a hideg akklimatizációs folyamatot. A távoli vörös fény nem mutatott ilyen hatást a jelen kísérleti rendszerben.

2) Mind a nagy fényintenzitás, mind a kiegészítő távoli vörös fény nagymértékben megnövelte a gamma-glutamilcisztein (a GSH szintézis közbenső terméke) és a ciszteinil-glicin (GSH bomlástermék) képződését a hidegkezelés során minden genotípusban, függetlenül azok fagytűrő képességétől. Ez a változás a □ESSE/□EC és a CySSGly/CysGly arányok nagymértékű csökkenését eredményezte, így mindkét tiol redukáltabbá vált.

3) A GSH tartalom hideg által kiváltott növekedését nem, vagy csak kis mértékben befolyásolta a nagy fényintenzitás vagy a kiegészítő távoli vörös fény. A GSSG tartalom és ennek következtében a GSSG/GSH arány azonban a hidegkezelés előtt nagymértékben csökkent a kiegészítő távoli vörös fényben a két fagyérzékeny genotípusban, de a két toleránsban nem volt ilyen változás. A távoli vörös fény csak a két érzékeny genotípusban indukálta nagymértékben a hmGSH és a hmGSSG felhalmozódást a hidegkezelés során. Ezek az eredmények a (hm)GSH metabolizmus fagytűréssel összefüggő szabályozását jelzik búzában.

4) Normál növekedési hőmérsékleten az antioxidáns enzimek kisebb aktivitását figyeltük meg nagy fényintenzitás mellett minden genotípusban az APX és a DHAR kivételével a CS-ben. Hasonló csökkenés csak a hidegkezelést követően következett be a másik két fényviszonyon. Ezek a megfigyelések a fény és a hőmérséklet antioxidáns enzimekre gyakorolt interaktív hatását mutatják.

5) Ellentétben az antioxidáns enzimek aktivitásának hideg által kiváltott csökkenésével normál intenzitású fehér fényben és nagyobb arányú távoli vörös fényben, több enzim génjének a transzkripciója fokozódott a hidegkezelés során. Így aktivitásuk nem transzkripciós szinten szabályozott.

6) Az ASA és a H_2O_2 jelentősen csökkentette a vizsgált fotoszintetikus paramétereket. A fotoszintézis zavara a lipidperoxidáció és az elektrolit- kiáramlás (membránkárosodás) hasonló mértékű növekedését eredményezte mindkét kezelés után.

7) Érdekes módon az endogén H_2O_2 szint magasabb volt az ASA exogén alkalmazása után, mint H_2O_2 kezelést követően. Ennek a különbségnek köszönhetően, az oxidált és a redukált aszkorbát és glutation arányát figyelembe véve, a redox környezet az ASA alkalmazását követően oxidáltabbá, a H_2O_2-kezelés után pedig redukáltabbá vált.

8) Az ASA gátolhatja, míg a H_2O_2 indukálhatja a redukálóerőt (NADPH-t) biztosító oxidatív pentóz-foszfát útvonalat és a glikolízist , amit a változatlan, illetve a csökkent glükóz-6-foszfát tartalom jelez. Ez a különbség magyarázatot adhat az ASA-kezelés után megfigyelt nagyobb H_2O_2-tartalomra.

9) A glikolízis, ezáltal a citrátciklus ASA révén történő gátlása, illetve ezek H_2O_2 általi indukciója felelős lehet a két biokémiai folyamat köztes termékeiből történő kisebb, illetve nagyobb mértékű aminosav-szintéziséért.

10) Az ASA kezelés után az oxidálóbb környezet számos, az ASA-GSH ciklushoz és a pentóz-foszfát útvonalhoz kapcsolódó transzkriptumot stimulált, ami hozzájárulhat az optimális redox környezet későbbi helyreállításához. Ezzel szemben a H_2O_2-kezelés után ez a hatás sokkal gyengébb volt, vagy nem is létezett.

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List of publications
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1. **Asghar, M. A.,** Balogh, E., Ahres, M., Szalai, G., Gondor, O. K., Darkó, É., ... & Kocsy, G. (2022). Ascorbate and Hydrogen Peroxide Modify Metabolite Profile of Wheat Differently. *Journal of Plant Growth Regulation*, 1-16. (**Q1, IF: 4.64**)
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Other publications

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Book chapters and conference proceedings

1. Hussain, M. I., Abideen, Z., Danish, S., **Asghar, M. A.,** & Iqbal, K. (2021). Integrated weed management for sustainable agriculture. *Sustainable Agriculture Reviews*, 52, 367-393. (**Book chapter**)

2. **Asghar, M. A.,** Balogh, E., Szalai, G., Gondor, O. K., ... & Kocsy, G. (2021). Redox-dependent adjustment of metabolites levels in wheat seedlings. *XII Congress of the Hungarian Society for Plant Biology, Hungary*. (**Conference proceeding**)

3. **Asghar, M. A.,** Balogh, E., Szalai, G., Gondor, O. K.,... & Kocsy, G. (2022). Modification of metabolite profile by ascorbate and hydrogen peroxide in wheat. *7th International Plant Phenotyping Symposium, The Netherlands*. (**Conference proceeding**)

Statement

As a supervisor of the Ph.D. work or/and the corresponding author of the following journal publications, I verify that all the results presented in this thesis and scientific publications were not used before to obtain any Ph.D. degree and will not be used in future as well:

Asghar, M. A., Balogh, E., Ahres, M., Szalai, G., Gondor, O. K., Darkó, É., ... & Kocsy, G. (2022). Ascorbate and hydrogen peroxide modify metabolite profile of wheat differently. *Journal of Plant Growth Regulation*, 1-16.

Asghar, M. A., Balogh, E., Szalai, G., Galiba, G., & Kocsy, G. (2022). Differences in the light-dependent changes of the glutathione metabolism during cold acclimation in wheat varieties with different freezing tolerance. *Journal of Agronomy and Crop Science*, 208(1), 65-75.

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